THESIS

IMPACT OF MICROBIAL INOCULANTS ON CADMIUM STRESS IN SELECTED LEGUMINOUS PLANTS

ABSTRACT

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ABSTRACT

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Abstract of the thesis submitted to the Aligarh Muslim University, Aligarh, India for the award of the degree of Doctor of Philosophy in Botany, 2015.

The present thesis comprises of six chapters

The importance of the problem and justifications for the present work undertaken were emphasized in chapter 1.

Chapter 2 is the review of the literature. It includes the literature available on the aspects of the physiological analysis of growth, biochemical characteristics, stress markers, components of antioxidant systems and yield of various crop plants under cadmium (Cd) stress. The importance of inoculation of *Rhizobium* and application of AM fungi (AMF) in the alleviation and in the regulation of plant growth and development under stress conditions were also reviewed. The chapter has been divided into sections and subsections for the better understanding of the work of other research reports in this field of study. In addition, the critical appraisal of the review of literature has also been included to identify the gap in the field of the study. The details of the material used in the study and the methodology adopted to determine various characteristics recorded in the four experiments were described in the chapter 3. In addition, the relevant information on the location of the study and the environmental conditions were also mentioned during the data sampling.

Chapter 4 included the results of the four experiments. Variation among leguminous crops for sensitivity and non-sensitivity were studied to select Cd sensitive and Cd non-sensitivite legumes. Details of physiological and biochemical processes occurring in Cd sensitive and Cd non-sensitive legumes and the role of microbes in regulating physiological processes under influence of microbes were studied. The data were statistically analyzed and level of significance was determined at $P<0.05$ using analysis of variance (ANOVA).

The results obtained in the Experiments were discussed in chapter 5 in the light of the observations recorded and supported with earlier findings, if available and also with the help of correlations. This chapter also presented the possible explanations of the data obtained to reach a conclusion and possible future aspects.

Chapter 6 presented the summary of the work reported in the thesis. A brief account of the importance of the study undertaken, experimental results and conclusions have been given below.

Importance of the study undertaken

Legumes are well known for their ability to fix atmospheric nitrogen (N) and enhance N pool of soil, leading to increase in crop productivity both in conventional or derelict soil. They provide pulses which are source of different amino acids, proteins and minerals in human diet. The vegetarian population, especially, relies on pulses for dietary protein requirements. They are used as food in a variety of forms besides this that they fix inert environmental N_2 symbiotically in association with root nodule bacteria and show no or little dependence on N fertilizers (Ma et al., 2006). The estimated amounts of N which can be fixed by leguminous crops range from 70-100 Kg ha^{-1} $year^{-1}$ in peas and beans to over 300 Kg ha^{-1} $year^{-1}$ for clover or lucernce (Postgate, 1982).

Plants are constantly exposed to adverse environmental conditions that negatively affect their growth and productivity. Heavy metal (HM) toxicity is one of the major abiotic stresses leading to hazardous effects in plants and alters physiological and metabolic processes (Villiers et al., 2011). Among HMs, Cd is one of the most toxic, a nonessential and mobile element present in soil that adversely affect plant growth and yield (Sanita di toppi and Gabbrielli, 1999, Qadir et al., 2004; Rahmanian et al., 2011). The toxicity of Cd in arable field is mainly due to the application of pesticides, industrial processes, fossil fuel combustion, cement manufacture and non-ferrous metal production, Cd-containing sewage sludge and phosphate fertilizers (Angelone and Bini, 1992; Sanita di toppi and Gabbrielli, 1999; Solis-Dominguez et al., 2007; Nazar et al., 2012, Sandalio et al., 2001). The high mobility of this metal in the soil-plant system makes its entrance easier into the food chain (Dalcorso et al., 2008). Although, Cd is not essential for plant growth, but it is readily taken up by roots and translocated into the leaves in many plant species

(Prasad, 1995). Despite the different mobility of Cd in plants, its accumulation in roots is more than other parts (Benavides, et al., 2005). Cadmium is not only immobilized in the root portion but also translocated to the aerial part (Yonis, 2007; Anjum et al., 2008). Physiological effects of Cd stress have been well documented in higher plants, especially those of agricultural importance such as legumes and cereals (Anjum et al., 2008; Farooqui et al., 2009).

The tolerance mechanisms in plant require the coordination of several complex physiological and biochemical processes to counter the detrimental effects of this contaminant (Hossain et al. 2012). Cadmium stress causes scattered signs of chlorosis and necrosis on the leaf lamina between the veins and marginal chlorosis in chickpea cultivars (Faizan et al., 2011). Cadmium toxicity causes decline in photosynthetic pigments, rubisco activity (Bibi and Hussain, 2005; Wani et al., 2008a, b) inactivation and denaturation of the enzymes, proteins, blocking of functional groups of metabolically important molecules, substitution or displacement of essential metal ions from biomolecules, conformational modification and disruption of membrane integrity (Ramesh, 2008; Villiers et al., 2011) which is finally attributed to altered activities of several key enzymes (Sharma and Dietz 2006). It also disturbs redox homeostasis indirectly by stimulating the formation of reactive oxygen species (ROS) and peroxidation of lipids. The tolerance mechanisms in plant require the coordination of several complex physiological and biochemical effects to counter the detrimental effects of this contaminant (Hossain et al., 2012) and plants have evolved a sophisticated antioxidant defense system such as superoxide dismutase, catalase and peroxidase (Maksymiec, 2007; Dube et al., 2009).

Nitrogen is a key macromolecule and earth can be conceived as immersed in an ocean of N. However, atmospheric N is not available to plants because of its relatively inert nature under normal temperature and pressure. The process of symbiotic N_2 fixation is of immense importance both from economic and environmental point of view. Mechanism of HM tolerance in *Rhizobium* are diverse and may involve energy-dependent efflux of the metal (Grass et al., 2000; Muson et al., 2000; Franke et al., 2001; Saltikov and Olson, 2002) precipitation of the metals as insoluble salts (Blake et al., 1993) alteration in membrane permeability to the metal (Levine and Marzluf, 1989) immobilization of the metal within the cell wall (Cervantes and Gutierrez-corona, 1994) production of chelating agents (Silver and

Phung, 1996) biochemical transformation of the metal ions (Williams and Silver, 1984) and immobilization, mobilization or transformation of metals to make them inactive (Nies, 1992). The synthesis of antioxidant enzymes in the inoculated legumes plays a pivotal role in protecting them from oxidative stress (Figueira et al., 2005; Corticeiro et al., 2006).

Maintenance of a healthy and pollution free environment is a current issue of global importance. The degradation of the soil by chemical fertilizers, fungicides, pesticides and weedicides has a chemophobia among the scientific community, all over in the world. Mycorrhiza is a term which designates a symbiotic non-pathogenic relationship between a group of fungi and plant roots. These fungi have emerged as potential biofertilizers, a cheap and environmental friendly alternative to expensive chemical fertilizers (Srivastava and Gupta, 1996). They increase the surface area available for absorption of water and nutrients which leads to a concomitant improvement in the uptake of nutrients such as P, Zn , Cu, Ca, K, Fe, Mg, Mn, Cl and N (Singh and Kapoor, 1999; Harrier, 2001) resulting in enhanced plant growth (Barea, 1991; Tarafdar and Kumar, 1996). Application of these fungi in soil can enhance the plants resistance to biotic and abiotic stresses (Abdel-Fattah et al., 2012 and Ruiz-Lozano, 2003; Asrar and Elhindi 2011) and reduce other soil stresses such as high salt levels, toxicities of mine spoils or landfills and HMs (Garg and Bhandari, 2014). Arbuscular mycorrhizal fungi can enhance plant resistance to Cd stress by improving the plants nutritional status particularly P uptake of plants and subsequently enhancing their growth (Janouskova and Pavlikova, 2010; Miransari, 2011a). Symbiosis of these findings can effectively bind the HMs through the chitin, free amino, hydroxyl and carboxyl group present in the cell wall (Joner et al., 2000a; Garg and Chandel, 2010) binding of metal in hyphae and metal sorption capacity of extraradical mycelium (Gohre and Paszkowski, 2006; Garg and Bhandari, 2014) precipitation and detoxification in soil matrix (Saraswat and Rai, 2011) adsorption of metal on the root surface or accumulation within root (Joner et al., 2000b; Garg and Chandel, 2010) dilution of metals by increased root or shoot growth (Mohammadi et al., 2011) and chelation by siderophores, metallothionein as well as polyphosphate granules (Upadhyaya et al., 2010). These fungi have genes which improve the capability of ROS scavenging and reduce Cd concentration in plants.

The positive synergistic interactions among components of the tripartite symbiotic association result in improved rates of N_2 fixation, P uptake and crop production under conditions of reduced N and P uptake (Azcon et al., 1991; Xavier and Germida, 2003; Faizan et al., 2004). However, the synergistic or additive interactions among the components of the tripartite association (*Rhizobium*-AMF-legume) under HM stress have not yet explored.

Considering the importance of legumes in maintaining soil fertility and conflicting reports on the effects of Cd on rhizospheric symbionts, some attentions have been focused to study the role of AM fungi and *Rhizobium* in plant metabolism and in the alleviation of Cd stress. Therefore, the present research work was undertaken with the following objectives.

- ✓ To screen and select legumes based on Cd sensitive grown in soil supplemented with different levels of Cd.
- ✓ To study the influence of *Rhizobium* application in the alleviation of Cd-induced effects in Cd least sensitive and Cd most-sensitive legumes.
- ✓ To study the application of AM fungi in the alleviation of Cd-induced effects in Cd least sensitive and Cd most-sensitive legumes.
- ✓ To study the influence of dual inoculation of *Rhizobium* and AM fungi in the alleviation of Cd-induced effects in Cd least sensitive and Cd most-sensitive legumes.

Experimental Results

The results of the experiments summarized below:

Experiment 1:

This experiment was performed to study the effect of five concentrations of Cd viz., 0, 25, 50, 75 and 100 mg Kg^{-1} soil on Cd accumulation in plants, stress markers, biochemical, growth and yield characteristics of five legume plants viz., methi (*Trigonella-foenum-graecum* L.; desi), broad bean (*Vicia faba* L.; VH-82-1), chick pea (*Cicer arietinum* L.; BG-472), pea (*Pisum sativum*; Kashi Udai) and lentil (*Lens culinaris*; Medik.; K-75). The treatments in this Experiment were arranged in a factorial randomized block design. Cadmium accumulation, stress markers, growth and biochemical characteristics were studied at pre-flowering (30DAS), flowering (60DAS) and post-flowering (90DAS) stages, while yield characteristics were noted

at harvest (120DAS). Tolerance index of five legumes was calculated and the plants were designed as Cd sensitive and Cd non-sensitive on the basis of their performance under Cd stress. The effect of Cd on growth, biochemical and yield characteristics was found significant at all sampling times. The increases in Cd levels decrease the growth, biochemical and yield characteristics of all the five plants at all sampling stages. The observations showed similar pattern of plants responses to Cd treatments. Maximum reduction in growth, biochemical and yield characteristics was noted with 100 mg Cd Kg^{-1} soil followed by 75, 50 and 25 mg Cd Kg^{-1} soil.

Among legumes, methi showed lesser decrease in growth, biochemical and yield characteristics followed by methi, broad bean, chick pea whereas, lentil and pea exhibited greater reduction in growth characteristics under Cd stress. The tolerance index of cultivars was methi> broad bean> chick pea> pea> lentil.

Experiment 2:

Experiment 2 was conducted on the basis of findings of Experiment 1. As observed in Experiment 1, Methi emerged as Cd non-sensitive and lentil as Cd sensitive. Among Cd levels, 100 mg Cd^{-1} Kg soil was found to be the most toxic and caused maximum reduction in characteristics studied. In the present Experiment, the aim was to study the alleviation potential of *Rhizobium* on the effects of 50 and 100 mg Cd Kg^{-1} soil. The treatments in this Experiment were arranged in a factorial randomized block design. The assessment was done by studying the changes in Cd accumulation in root and shoot, stress markers (MDA and proline content), growth, biochemical characteristics, components of antioxidant defense system and yield characteristics of non-sensitive (methi) and sensitive (lentil) plants. The sampling for Cd accumulation, growth and biochemical characteristics, stress markers, components of antioxidant defense system were recorded at pre-flowering (30DAS), flowering (60DAS), and post-flowering (90DAS) stages. The yield characteristics were noted at harvest (120 DAS). The growth, biochemical characteristics and yield decreased maximally in both the plants treated with 100 mg Cd Kg^{-1} soil. Contrarily, there was significant increase in the Cd accumulation, MDA, proline content and components of enzymatic antioxidant system in sensitive plant. Lentil exhibited greater effects than non-sensitive plant i.e. methi. The application of *Rhizobium* in soil maximally alleviated the toxic effects of 50 mg Cd Kg^{-1} soil compared to 100 mg Cd Kg^{-1} soil and

improved growth, biochemical characteristics, stress markers, components of antioxidant defense system and thus yield characteristics of both the plants. The alleviation effect of *Rhizobium* was higher in methi than lentil.

Experiment 3:

Experiment 3 was conducted on the basis of the findings of Experiment 1. In Experiment 2, it was observed that the application of *Rhizobium* to plants treated with 50 mg Cd Kg^{-1} soil alleviated Cd-induced toxicity in both the plants. Inoculation of *Rhizobium* maximally overcome the toxic effects of 50 mg Cd Kg^{-1} soil in methi (non-sensitive plant) while, the same microbe lowered the Cd-induced toxic effects in lentil to some extent. The present experiment was aimed to study the effect of application of AM fungi for alleviation of adverse effects of 50 mg Cd Kg^{-1} soil and 100 mg Cd Kg^{-1} soil. The treatments in this Experiment were arranged in a factorial randomized block design. The assessment was done by analyzing the changes in Cd accumulation in root and shoot, growth and biochemical characteristics, stress markers, components of antioxidant defense system recorded at pre-flowering (30DAS), flowering (60DAS), and post-flowering (90DAS) stages and yield characteristics was done at harvest.

The alteration in Cd accumulation, growth, biochemical characteristics, stress markers, components of antioxidant defense system and yield characteristics caused by 50 mg Cd Kg^{-1} soil were alleviated by AM fungi in methi (non-sensitive plant) and lentil (sensitive plant) but the alleviation potential of AM fungi varied between plants. Application of AM fungi not only ameliorated the Cd-induced effects but also increased growth, biochemical characteristics, components of antioxidant defense system and thus yield characteristics in methi. In lentil, application of this symbiont only lowered the adverse effects of Cd.

Experiment 4:

Experiment 4 was conducted on the basis of the findings of Experiment 1, Experiment 2 and 3, it was observed that the application of *Rhizobium* and AM fungi singly to plants treated with 50 mg Cd Kg^{-1} soil alleviated Cd-induced toxicity in both the plants. Inoculation of both the symbionts maximally overcome the toxic effects of 50 mg Cd Kg^{-1} soil in methi (non-sensitive plant) while, the same microbes lowered the Cd-induced toxic effects in lentil to some extent but it was more than their single inoculation. The present experiment was aimed to study the effect of dual inoculation

of *Rhizobium* and AM fungi for alleviation of adverse effects of 50 mg Cd Kg^{-1} soil and 100 mg Cd Kg^{-1} soil. The treatments in this Experiment were arranged in a factorial randomized block design. The assessment was done by analyzing the changes in Cd accumulation in root and shoot growth, biochemical characteristics, stress markers, components of antioxidant defense system were recorded at pre-flowering (30DAS), flowering (60DAS), and post-flowering (90DAS) stages and yield characteristics was done at harvest. The alteration in Cd accumulation, growth, biochemical characteristics, stress markers, components of antioxidant defense system and yield characteristics caused by 50 mg Cd Kg^{-1} soil were alleviated by in methi (non-sensitive plant) and lentil (sensitive plant) but the alleviation potential of co-inoculation both the symbionts varied between plants. Combined application of *Rhizobium* and AM fungi not only ameliorated the Cd-induced effects but also increased growth, biochemical characteristics, components of antioxidant defense system and thus yield characteristics in methi. In lentil, application of these symbionts lowered the adverse effects of Cd. But the extent of decrease was more than their single inoculation.

The present chapter is followed by an up-to-date bibliography of the literature cited in the text.

CONCLUSIONS

Conclusively, symbiotic rhizospheric microbes are known to decipher essential role in plant metabolism and augmenting growth and productivity of crops. However, a combination of symbionts is required for sustainable agriculture to avoid chemical fertilizers under varied environmental conditions. Legumes display their inherent potential. Methi surpassed other legumes tested and tolerated Cd stress to a significant degree. Lentil was weak in performance and least tolerant to Cd stress among the legumes tested. Therefore, methi exhibited lesser decreases in growth, biochemical and yield characteristics under Cd stress. Correspondingly, methi showed lesser oxidative stress and increased antioxidant system than lentil to protect photosynthetic machinery and consequent effects on its attributes. *Rhizobium* and AM fungi proved significant potential in the alleviation of Cd stress in both the plants. The decreases in the characteristics observed due to Cd stress were lowered by the application of *Rhizobium* and AM fungi. The co-inoculation of *Rhizobium* and AM fungi not only

resulted in restricting the decrease caused by Cd but also nullified the characteristics values over the control. A combined package of *Rhizobium* and AM fungi appears to be most effective in the cultivation of legumes under Cd stress. The effect of this combination was due to the co-ordination of *Rhizobium* and AM fungi in maintaining plant metabolism and alleviating Cd stress.

New reports in the thesis and future prospects

Rhizospheric microbes play a critical role in increasing plants resistance to environmental stress. They not only help in the uptake and assimilation of important plant nutrients in higher plants, and are crucial factors in determining plant growth, vigour and crop yield, but they also alleviate the toxicity and stress caused due to HM. The observations of pot experiments recorded meticulously over three years have no doubt established the alleviation potential of individual as well as combined effects of *Rhizobium* and AM fungi to mitigate the inhibitory effects of Cd on legumes. No effort has been made up till now to study the synergistic and additive impact of these rhizospheric microbes on growth and development of crops under Cd stress. The effect of combined application of *Rhizobium* and AM fungi on oxidative stress and antioxidants under Cd stress has been reported for the first time. The combined application of *Rhizobium* and AM fungi protocol can be recommended to farmers to increase the productivity of legumes under Cd stress.

In addition, an attempt may be made to record the following observations that could not be undertaken due to limited facilities and time.

- To study the mechanism (s) of regulation of symbionts at the molecular level in crop plants under varied environmental conditions.
- To manipulate steps of pathways leading to the production of enzymatic antioxidant (Ascorbate peroxidase, Glutathione reductase and Monodehydro-ascorbate reductase Ascorbate peroxidase, Glutathione S-transferase), non-enzymatic antioxidant (Ascorbic acid, Glutathione), estimation of H_2O_2 and extent of membrane damage.
- To understand the Cd-induced stress response modulated by *Rhizobium* and AM fungi at molecular and mechanistic levels. This would help to develop an effective strategy to raise transgenic species for stress resistance.
- Same Experiments can be carried out in field conditions.
- Nitrogenase activity for assessing the N_2 fixation ability of legumes can be done.

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IMPACT OF MICROBIAL INOCULANTS ON CADMIUM STRESS IN SELECTED LEGUMINOUS PLANTS

THESIS

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IN

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2015

Dedicated

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Certificate

This is to certify that the thesis entitled **"Impact of Microbial Inoculants on Cadmium Stress in Selected Leguminous Plants"** submitted in partial fulfillment of the requirements for the degree of **Doctor of Philosophy** in Botany, is a faithful record of the bonafide research work carried out at Aligarh Muslim University, Aligarh by **Ms. Rubina Perveen** under my guidance and supervision and that no part of it has been submitted for any other degree or diploma.

Shahla Faizan

(Dr. Shahla Faizan)

Supervisor

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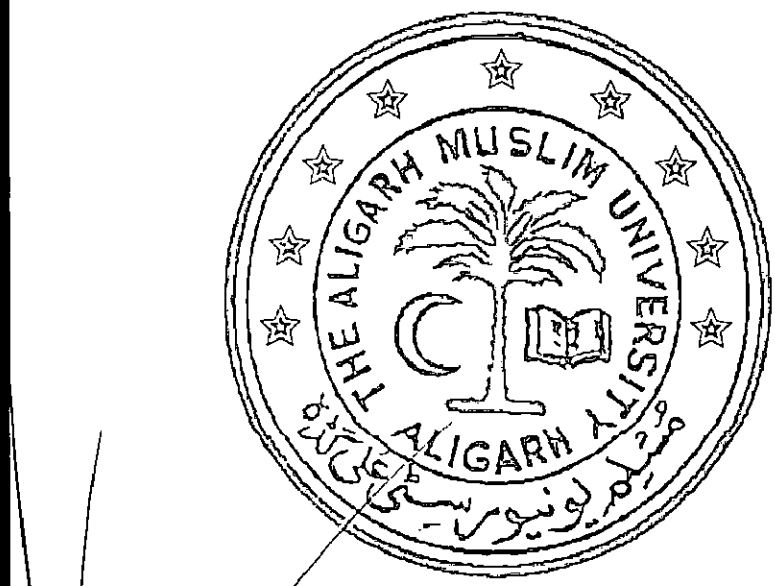
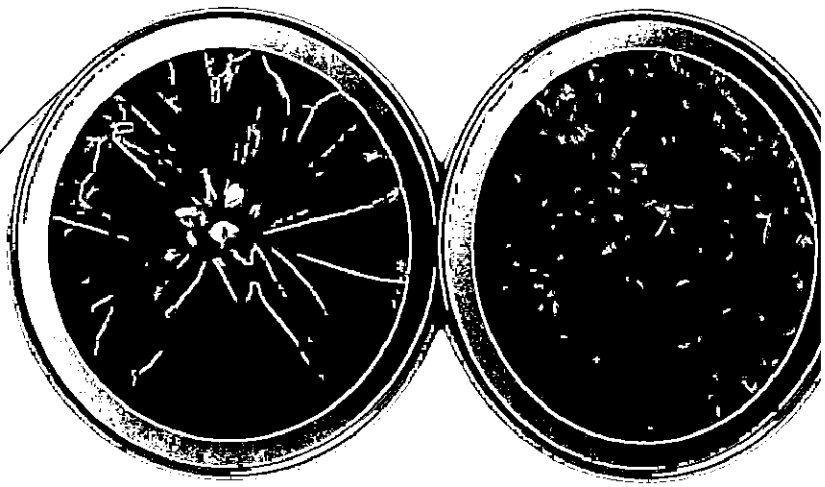
Chapter-1

Introduction





INTRODUCTION

Legumes are well known for their ability to fix atmospheric nitrogen (N) and enhance N pool of soil, leading to increase in crop productivity both in conventional or derelict soil. They provide pulses which are source of different amino acids, proteins and minerals in human diet. The vegetarian population, especially, relies on pulses for dietary protein requirements. They are used as food in a variety of forms, besides, this that they fix inert environmental N symbiotically in association with root nodule bacteria and shows no or little dependence on N fertilizers (Ma et al., 2006). They are able to grow under a wide range of climatic conditions and edaphic factors such as soil moisture, toxicants, pH and texture etc. The estimated amounts of N which can be fixed by leguminous crops range from 70-100 Kg ha^{-1} $year^{-1}$ in peas and beans to over 300 Kg ha^{-1} $year^{-1}$ for clover or lucernce (Postgate, 1982). Excessive metal concentration on the other hand causes undeniable damage to *Rhizobium*, legumes and their symbiosis.

Plants are constantly exposed to adverse environmental conditions that negatively affect their growth and productivity. Heavy metal (HM) toxicity is one of the major abiotic stresses leading to hazardous effects in plants and alters physiological and metabolic processes (Villiers et al., 2011). They become toxic when their concentration exceeds a beyond certain threshold level which further depends upon plant species, edaphic conditions and the type of the metal. Among HMs, Cadmium (Cd) is one of the most toxic, nonessential and mobile element present in soil that adversely affect plant growth and yield. (Sanita di toppi and Gabbrielli, 1999; Qadir et al., 2004; Rahmanian et al., 2011). The toxicity of Cd in arable field is mainly due to the application of pesticides, industrial processes, fossil fuel combustion, cement manufacture, non-ferrous metal production, Cd-containing sewage sludge and phosphate fertilizers (Angelone and Bini, 1992; Sanita di toppi and Gabbrielli, 1999; Solis-Dominguez et al., 2007; Nazar et al., 2012, Sandalio et al., 2001). The high mobility of this metal in the soil-plant system makes its entrance easier into the food chain (Dalcorso et al., 2018). Although, Cd is not essential for plant growth, but it is readily taken up by roots and translocated into the leaves of many plant species (Prasad, 1995). The degree to which plants are able to take up Cd by their roots depends on its concentration in the soil and its bioavailability,

iulated by the presence of organic matter, pH, redox potential, temperature and centration of other elements (Eriksson, et al., 1996; Ciecko, et al., 2001; Yu et al., 5; Singh et al., 2008). Despite the different mobility of Cd in plants, its umulation in roots is more than other parts (Benavides, et al., 2005). Cadmium is only immobilized in the root portion but also translocated to the aerial part (Yonis, 7; Anjum et al., 2008). Physiological effects of Cd stress have been well umented in higher plants, especially those of agricultural importance such as umes and cereals (Anjum et al., 2008; Farooqui et al., 2009). The tolerance hanisms in plant require the coordination of several complex physiological and hemical processes to counter the detrimental effects of this contaminant (Hossain l., 2012). Legumes and cereals show visual symptoms like reddish brown spots on nary and secondary leaves such as blackening, browning, burning and weakening tems and leaves. (Anjum et al., 2008; Farooqi et al., 2009). Cadmium stress causes tered signs of chlorosis and necrosis on the leaf lamina between the veins and ginal chlorosis in chickpea cultivars (Faizan et al., 2011). The reduction in wth, biomass and yield with increased levels of Cd has been primarily attributed to turbed photosynthesis (Verma and Dubey, 2002; Wahid et al., 2007) which may be to the decline in photosynthetic pigments and rubisco activity (Bibi and Hussain, 5; Wani et al., 2008b). Cadmium toxicity results in inactivation and denaturation he enzymes, proteins, blocking of functional groups of metabolically important lecules, substitution or displacement of essential metal ions from biomolecules, formational modification and disruption of membrane integrity (Ramesh, 2008; liers et al., 2011) which is finally attributed to altered activities of several key ymes (Sharma and Dietz 2006; Dubey, 2011). It also disturbs redox homeostasis irectly by stimulating the formation of reactive oxygen species (ROS) and oxidation of membrane lipids.

The tolerance mechanisms in plant require the coordination of several nplexes physiological and biochemical effects to counter the detrimental effects of (Hossain et al. 2012) and plants have evolved a sophisticated antioxidant defense tem to scavenge them. Among antioxidant enzymes, superoxide dismutase istitutes the primary step of cellular defense and dismutates superoxide radicals to lrogen peroxide and oxygen. Accumulation of hydrogen peroxide which is a strong

oxidant is prevented in cell by catalase and peroxidase (Maksymiec, 2007; Dube et al., 2009).

Nitrogen is a key macromolecule and earth can be conceived as immersed in an ocean of N. However, atmospheric N is not available to plants because of its relatively inert nature under normal temperature and pressure. The process of symbiotic N_2 fixation is of immense importance both from economic and environmental point of view. Industrial fixation of atmospheric N needs great energy inputs and is not environment friendly because of the attendant risk of leakage of ammonia into the atmosphere.

A dramatic change in microbial composition and their activity occur in metal-enriched soil which adversely affects (Khan et al., 2009a; Krujatz et al., 2011) nutrient pool, soil fertility, symbiosis and yield of legumes like *Trifolium pratense* (McGrath et al., 1988), *Cicer arietinum* (Hayat, 2011; Wani et al., 2008b; Wani and Khan, 2010), *Vigna radiata* (Wani et al., 2007a) and *Pisum sativum* (Wani et al., 2008a). There are numerous reports where alleviated Cd level limits the rhizobial growth, their host legume, the total soluble protein content and N fixation to concomitantly reduce the crop yield (Moftah, 2000; Broos et al, 2005; Figueira et al., 2005). Mechanisms of HM tolerance in *Rhizobium* are diverse and may involve energy-dependent efflux of the metal (Grass et al., 2000; Muson et al., 2000; Franke et al., 2001; Saltikov and Olson, 2002), precipitation of the metals as insoluble salts (Blake et al., 1993), alteration of membrane permeability for the metal (Levine and Marzluf, 1989), immobilization of the metal within the cell wall (Cervantes and Gutierrez-corona, 1994), production of chelating agents (Silver and Phung, 1996) biochemical transformation of the metal ions (Williams and Silver, 1984) and immobilization or transformation of metals to make them inactive (Nies, 1992). The synthesis of antioxidant enzymes in the inoculated legumes plays a pivotal role in protecting them from oxidative stress (Figueira et al., 2005; Corticeiro et al., 2006).

Maintenance of a healthy and pollution free environment is a current issue of global importance. The degradation of the soil by chemical fertilizers, fungicides, pesticides and weedicides has a chemophobia among the scientific community, all over the world. Mycorrhiza is a term which designates a symbiotic non-pathogenic relationship between a group of fungi and plant roots. These fungi have emerged as

ntial bio-fertilizer, a cheap and environmental friendly alternative to expensive nical fertilizers (Srivastava et al., 1996) and the greatest advantage given by them ost plant is many fold increase in surface area available for absorption of water nutrients which leads to a concomitant improvement in the uptake of nutrients as P, Zn , Cu, Ca, K, Fe, Mg, Mn, Cl and N (Harrier, 2001; Singh and Kapoor, 9) resulting in enhanced plant growth. (Barea, 1991; Tarafdar and Kumar, 1996). lication of these fungi in soil can enhance the plants resistance to biotic and tic stresses (Asrar and Elhindi, 2011; Abdel-Fattah et al 2002; Ruiz-Lozano, 3) and reduce other soil stresses such as high salt levels, toxicities of mine spoils andfills and HMs (Garg and Bhandari, 2014). Arbuscular mycorrhizal fungi can ance plant resistance to Cd stress by improving the plant's nutritional status icularly P uptake and subsequently enhancing its growth (Janouskova and likova, 2010; Miransari, 2011a). Symbiosis of this fungi can effectively immobile HMs through the chitin, free amino, hydroxyl and carboxyl groups present in the wall (Joner et al., 2000; Garg and Chandel, 2010), binding of HM to the hyphae metal sorption capacity of extraradical mycelium (Gohre and Paszkowski, 2006; g and Bhandari, 2014), precipitation and detoxification in soil matrix (Saraswat Rai, 2011), adsorption of metal on the root surface or its accumulation within t (Joner et al., 2000; Garg and Chandel, 2010), dilution of metal by increased root shoot growth (Mohammadi et al., 2011) and chelation by siderophores, allothionines as well as polyphosphate granules (Upadhyaya et al., 2010). uscular mycorrhizal fungi carry genes which improve the capability of ROS venging and reduce Cd concentration in plants. In general, several eco-icological adverse effects of HMs on plants and soil microorganisms have been l reported with varying degree of effects on legumes.

The positive synergistic interactions among components of the tripartite ibiotic association result in improved rates of N_2 fixation, phosphorus uptake and p production under conditions of reduced N and P uptake (Azcon et al., 1991; vier and Germida, 2003; Faizan et al., 2004). However, the synergistic or additive eractions among the components of the tripartite association (*Rhizobium*-AMF-ume) under HM stress have not yet explored.

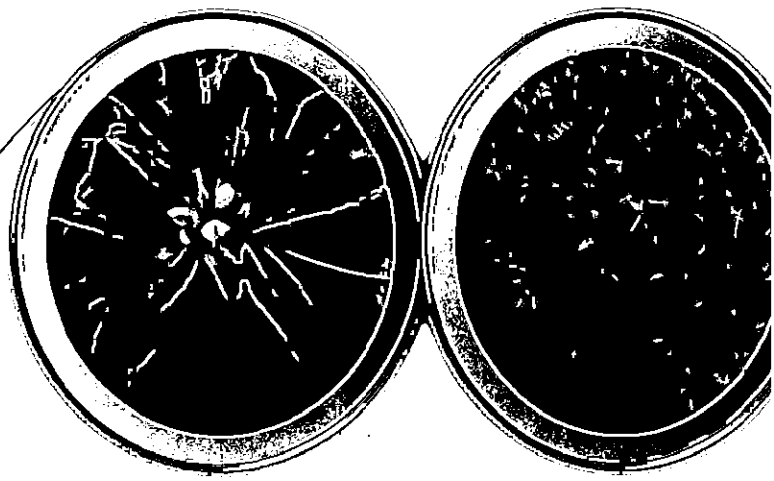
Considering the importance of legumes in maintaining soil fertility and iflicting reports on the effects of Cd on rhizospheric symbionts, some attentions

have been focused to study the role of AM fungi and *Rhizobium* in plant metabolism and in the alleviation of Cd stress. Therefore, the present research work was undertaken with the following objectives.

- ✓ To screen and select legumes based on Cd sensitive grown in soil supplemented with different levels of Cd.
- ✓ To study the influence of *Rhizobium* application in the alleviation of Cd-induced effects in Cd least sensitive and Cd most-sensitive legumes.
- ✓ To study the application of AM fungi in the alleviation of Cd-induced effects in Cd least sensitive and Cd most-sensitive legumes.
- ✓ To study the influence of dual inoculation of *Rhizobium* and AM fungi in the alleviation of Cd-induced effects in Cd least sensitive and Cd most-sensitive legumes.

Chapter-2

Review of Literature



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REVIEW OF LITERATURE

In the successful era of Green Revolution, efforts were made to increase the productivity with the involvement of multiple factors. The primary focus in this aspect was the use of inorganic fertilizers, irrigation and high yielding cultivars. In an efforts of achieving the goal of enhancing crop productivity with the use of chemical fertilizers and waste water irrigation, a variety of abiotic stress factors limiting crop productivity have been added simultaneously. Use of excess phosphate fertilizers sufficiently adds Cadmium (Cd) in soil up to the toxic level for plant and ecosystem. Beside this, various other heavy metals (HMs) have been added during the course of agricultural activities.

Legumes belong to family *Fabaceae,* are well known for their ability to fix atmospheric nitrogen (N), to enhance soil N, increase growth and yield especially both in conventional or derelict soils and constitute a major portion of human food throughout the world. The symbiotic relationship between rhizobia and legumes provides nutrients to plants, facilitate their growth and restores derange ecosystem (Abd-Alla et al., 1999). They help in crop rotation, control weeds, diseases and insects, as well as improve soil texture and fertility. These characteristics together make them a valuable crop to provide pulses, fibers, timber, raw materials of many products and forage.

Trigonella foenum-graecum L. is commonly known as fenugreek or methi. It is an annual crop grown in winter season in India. It is rich in calories, seed proteins, B-vitamins and minerals. Major fenugreek producing countries are India, Pakistan, Afganistan, Bangladesh, Argentina, Egypt, France, Yemen, Spain, Turkey, Morocco and China. India is the largest producer of this in the world (Zohary and Hopf, 2000). In India, fenugreek has medicinal importance and used as vegetables as well as culinary purpose. They play important role in the preparation of phytoestrogens and diosgenins to fight breast cancer and in the reduction of serum cholesterol (Amin et al., 2009).

Vicia faba, is also known as broad bean is the most cultivated food crop of the world covering 25% of total cultivated area (Aouar-sadli et al., 2008). It is also rigid and erect plant that can tolerate harsh climates. This plant is grown in Iran, Egypt,

Morocco, Sicily, Sudan, Greece, Ethopia, Nepal, Peru and Colombia. It has high nutritional value and is economic and eco-friendly vegetable crop. It is rich in tyramine, contains alkaloids vicine, convicine, L-dopa and condensed tannins, besides it also has a good amount of carbohydrates, protein and dietary fiber.

Cicer arietinum or chickpea is also known as Bengal gram. It is also called as Egyptian pea or chana. Seeds are high in protein. It is one of the earliest cultivated legumes. Three main kinds of chickpea are desi, Mumbai or kabuli. Desi chickpea has higher fiber content than Kabuli and hence have very low glycemic index, which makes it suitable for diabetic people. Chickpeas are grown in the Mediterranean, Western Asia, the Indian subcontinent, Australia, the Palouse region, and the Great Plains. India is the leading producer of gram and produces nearly fifteen times more than the second-largest producer, Australia.

Pisum sativum is also known as garden pea. It is an annual vine herb grown in winter. It was originated from Mediterranean basin and North East and grown in many parts of the world. Seeds are used for vegetable and are rich in fiber, protein, vitamins, folate, minerals and lutein (yellow carotenoid pigment). Dry weight has about one-quarter protein and one-half carbohydrate which is mostly sugar. Peptide fractions of pea seed have an ability to chelate metals and inhibit linoleic acid oxidation.

Cicer arietinum is drought resistant and can be grown in water logged and saline soil and nutrient deficient soil (Muehlbaur et al., 2002). Its seeds contain protein (26%), carbohydrate (6%) and also have higher calories compared to other legumes. It also contains certain amino acids such as methionine and cystine Muehlbauer et al., 2002) vitamin B and minerals etc.

Divalent cationic HMs may be essential for various metabolic activities of plants and microbes including rhizobia at very lower concentrations (Arora et al., 2009; Mandal and Rabindranath, 2012). At higher concentrations they induce toxicity symptoms in plants. Nonessential HMs, however, pose greater survival threat, reduce growth, crop productivity and contaminate the food products (Salt et al., 1995; Akinola and Ekiyoyo, 2006). Cadmium is a readily available nonessential HM with high mobility and enrichment ratio in plant tissues. Excess Cd concentration in soil causes undeniable damage to rhizobia, legumes and their symbiosis. Its excess

concentration along with other HMs affects the survival and ability of rhizobia to form N_2-fixing nodules (Arora et al., 2009; Corticerio et al., 2012). Plants have been screened on the basis of several internal defense mechanisms to resist Cd-induced toxicity to manage their growth and productivity.

Plants also apply different types of avoidance mechanisms such as they retain Cd in soil or in root, efflux excess metal or defoliate high Cd containing lower leaves. Cultivation of legumes with other crops is reported to increase Cd contamination in the neighbouring crops in Cd polluted areas (Liu et al., 2012; 2013). The identification of mechanisms that improve rhizobial tolerance to Cd and its ability to improve nodulation efficiency with rhizobia in metal-contaminated soil is an important issue that requires urgent attention for maintaining soil fertility in metal polluted lands. Symbiotic relationships of arbuscular mycorrhiza (AM) fungi with roots are important for plant growth, phosphorus (P) acquisition, productivity and tolerance of plants in metal contaminated soil. Its colonization with the roots sieve nonessential HM or excess of essential HM uptake and increase plants access to other minerals especially P and water under Cd stress (Garg and Bhandari, 2014). Beside N fixation, rhizobia have evolved several mechanisms to circumvent metal stress in *Rhizobium*-legume symbiosis in HM-contaminated sites (Khan et al., 2012; Takacs, 2012).

2.1 Heavy metals, Cd and biological functions

The term HM includes metals and metalloids with the density greater than 5 g/ml. Some mono and divalent cations may be essential, nonessential or beneficial mineral elements. Essential elements act as co-factors of many enzymes as well as signaling molecules and are required in several metabolic processes and follow bell-shaped-relationship of toxicity (Marshner, 2012; Fig. 1).

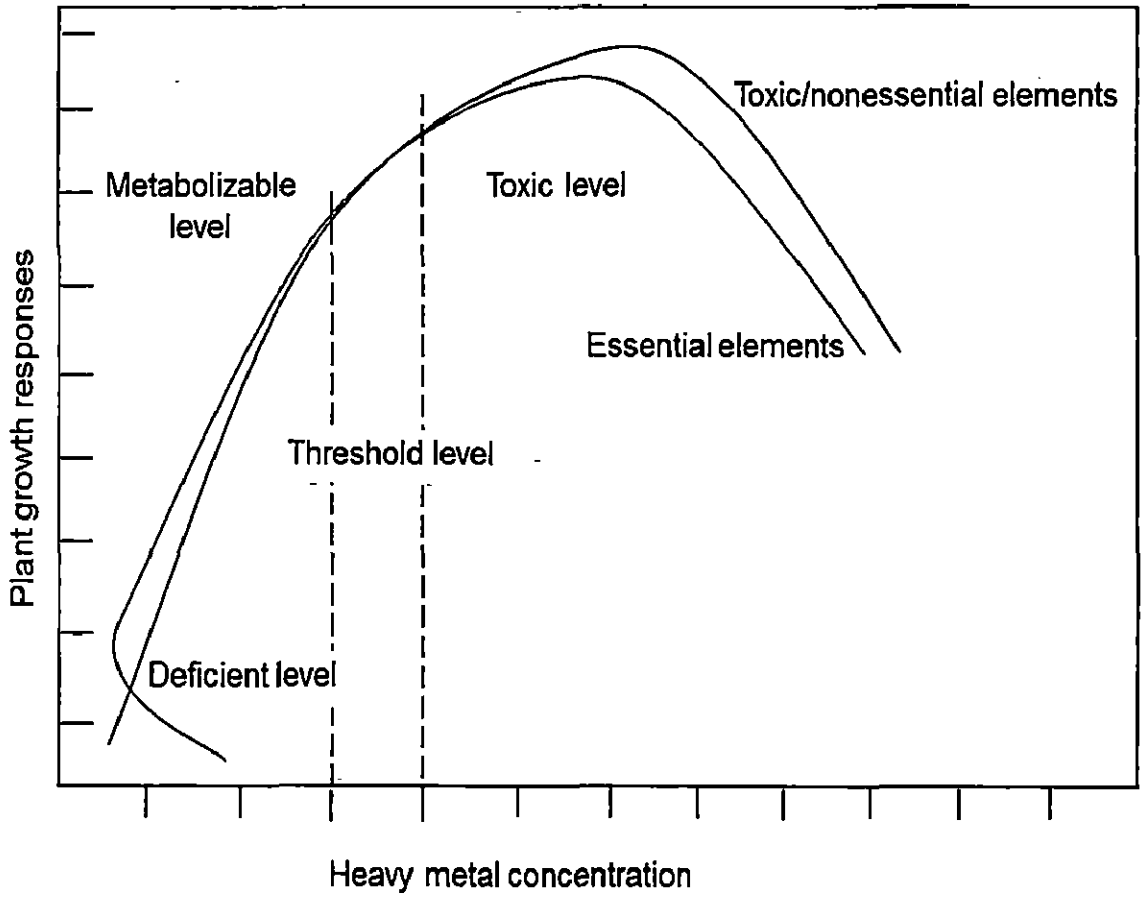


Figure 2.1: Bell shaped curve for plant responses to heavy metals uptake

The nonessential heavy metals are a matter of concern because they accelerate the rate of mortality, reduce survival potential of plants and induce toxicity symptoms in them. These HMs are not vital for plant growth, hence are considered as nonessential elements (Velaiappan et al., 2002). Recently, another class of HMs has been recognized, which do not have direct role in metabolism, but their presence significantly improves health and immunity of the plants. They are categorized as beneficial HMs. The role of *Co and †Se is disputed among physiologists to considered them either essential or not. The table appended below shows the types of HMs/minerals based on the essentiality criteria in plants with their biological roles (Table 1).

Table 2.1: Classification of metal(loid)s and their roles in plant system

S.No.	Non-essential	Biological significance
1.	Pb, Cd, Hg, Al, Cr, As, U & Ti	No biological role. Toxic after a threshold tolerance level
	Essential	**Biological significance**
1.	Fe (iron) Fe^{2+} or Fe^{3+}	In chlorophyll synthesis, component of cytochromes, ferridoxin catalase, peroxidase, nitrogenase, photosynthetic and respiratory chain & leg-hemoglobin
2.	Zn (zinc) Zn^{2+}	In auxin biosynthesis, component of several enzymes or activator (e.g. carbonic anhydrase) and maintains ribosomal structure
3.	Mo (molybdenum) MoO^{4+}	In nitrogen fixation and nitrate reductase reduction (nitrate reductase)
4.	B (boron) BO_3^{3-} or $B_4O_7^{2-}$	Absorption of calcium, role in root nodulation, formation of cell wall
5.	Cu (copper) Cu^{2+} or Cu^{2}	Part of enzymes (e.g. Superoxide dismutase), participates in several oxidation-reduction reactions of electron transport
6.	Mn (manganese) Mn^{2+}	Part of enzymes (e.g.), oxygen evolution in photosynthesis, chloroplast integrity
7.	Mo (molybdenum) MoO_2^{2+}	Part of nitrogenase; nitrogen fixation, in conversion of inorganic phosphate to organic form
8.	Ni (nickel) Ni^{2+}	Nitrogen (urieds) metabolism, its role is disputed among physiologists
	Beneficial	**Biological significance**
9.	*Co (cobalt) Co^{2+}	Part of nitrogenase; nitrogen fixation
10.	†Se (selenium) Se^{6+}, Se^{4+}	Improves antioxidant activity
11.	Ca (calcium) Ca^{2+}	In secondary signaling, cell-wall formation, photosynthates allocation, microbial activity stimulation, also work as enzyme activator
12.	Na (sodium) Na^{2+}	Regeneration of phospho-enol pyruvate in CAM and C_4 plants. It could substitute K in certain cases.
13.	Va (vanadium)	Essential for green algae
14.	Si (silicon)	Cell wall formation, prevents cuticular water loss, improves plant structural defense

Industrial activities and sewage sludge application have largely contributed to the buildup of HMs in the terrestrial environment (Viti et al., 2003). Problem of pollution by these metals on land, in water and soil may pose serious threat to human

health and ecological balance (Sundar et al., 2010). Metals ultimately get contaminated and accumulated in the ground water which ultimately leads to serious aggravation of the problem (Malarkodi et al., 2007). Exposure of HMs results in acute and chronic toxicity in human beings and interferes with the functions of kidney, liver and lungs (Alsaleh et al., 2006). The increasing influx of HMs into water bodies from industrial, agricultural, and domestic activities is of global concern because of their well-documented negative effects on human and ecosystem (Khan et al., 2008; Govind and Madhuri, 2014). The toxicity of HMs is a problem from ecological, evolutionary and environmental reasons (Nagajyoti et al., 2008).

2.1.1 Sources of heavy metals

Heavy metals are significant environmental pollutants (Jarup, 2003; Nagajyoti, 2010) and their availability in soil depends not only on natural processes which are specially lithogenic and pedogenic but also on anthropogenic factors such as mining, combustion of fossil fuels, urban waste disposal, soil runoff, metal working industries, boating activities, phosphate fertilizer application, sewage treatment plant effluents, and municipal solid waste disposal sites (Table 2). The presence of these metals in industrial and urban waste water is one of the main causes of water and soil pollution (Wang et al., 2005). They have adverse effects on ecosystem and human health (Srivastava and Thakur, 2006). Tremendous increase in the use of these metals over the past decades has inevitability resulted in an increased flux of metallic substances in the environment. Some metal ions are cumulative poisons capable of being assimilated and stored in the tissues of organisms causing noticeable adverse physiological effects (Garg et al., 2007).

Application of sewage sludge to agricultural land increases the level of HMs in soil which is attributed to the effect of different factors such as soil properties or different agricultural practices (McBride, 2002; Delgado Arroyo et al., 2002; Bettiol and Ghini, 2011; Usman et al., 2012). Household municipal and industrial wastes are also sources of different HMs in to the soil (Goel, 2006; Bundela et al., 2010) and their contamination above the permissible limit leads to decline in agricultural yield (Salt and Rauser, 1995; Akinola and Ekiyoyo, 2006). Their accumulation in the environment becomes a major cause of environmental pollution. Among various HMs, Cd has received much attention regarding its adverse effect on growth of plants as well as health of human, cattle and also on ecosystem.

Table 2.2: Sources of heavy metals pollution

Types	Sources	References
Natural sources	Emissions	EMEP/EEA, 2009; 2010
	Transport of continental dust	Crusius et al., 2011
	Withering of metal enriched rocks	Kimball et al., 2010
Anthropogenic sources	Agro-chemicals	Dragovic et al., 2008
	Waste disposal	Furgusson and Kim, 1991
	Industries	Adelekan and Abegunde, 2011
	Atmospheric deposition	Batisani and Yamal, 2010
	Smelting and mining	Cortez et al., 2010

2.1.2 Cadmium

2.1.2.1 Chemistry of cadmium

Cadmium was discovered by Strohmeyer in 1817. It is a soft, hazardous HM that occurs naturally in earth's crust. It has been placed in II-B group and 5th period of the periodic table and is located at the end of the second row of transition elements with atomic number being 48, atomic weight 112.4, density 8.65 g cm^{-3}, melting point 320.9°C, and boiling point 765°C, Hg, Pb and Cd are three HMs which poisonous and are not known for any essential biological function. It falls under the category of non-essential divalent cations which is the most abundant and readily available in plant body and has toxic effects in aerial parts of plants (Kabata-Pendias, 2011).

2.1.2.2 Soil cadmium level and its uptake in 'producers'

Cadmium is a non-essential element and one of the most hazardous HMs that exist in the polluted field. Industrial revolution has brought about a fast spread of it throughout the world and its increased build-up in the soil has polluted the environment (FWPCA, 1968). Its ions are readily taken up by the roots and translocated to the above-ground vegetative parts (Shamsi et al., 2008). The permissible limit of Cd in soil ranges from 0.04 mM to 0.32 mM. Beyond this level of Cd in soil, the yield of crop decreased and the quality of field products get degraded (Hassan et al., 2005). The uptake pattern also depends upon the dose of Cd salt and the plant species raised in such soil. Cereals and vegetables are the most susceptible to

increased contamination through raised levels of Cd in the soil, the range of variation of Cd for cereal grains is from 0.013 to 0.22 mg Kg^{-1}, for grasses range is from 0.07 to 0.27 mg Kg^{-1} soil and whereas legumes crops vary from 0.08 to 0.28 mg Kg^{-1} soil (Kabata-Pendias and Pendias, 2001). In leafy and fruit vegetables, Cd is reported to be 0.6 μg/g tissue fresh mass (Sharma et al., 2009). It is not only toxic for plant metabolism and growth but also its enrichment ratio is more compared to the other toxic HMs. The mycorrhizal associations have been shown to increase, decrease or have no effect on metal uptake of plants. However, rhizospheric population of AM and *Rhizobium* also detoxify Cd by the exudation of several metal chelators e.g. siderophores and organic acids.

Plants also exude organic acids to detoxify the active valance electron of free metal ions in the soil and cytoplasm. The ratio of translocation factor from soil to plant and transportation factor from root to shoot depends upon several avoidance or resistance mechanisms offered by the plants alone or in association with other crops, soil animals or soil microbiota.

2.1.2.3 Toxicity effects of cadmium on ecosystem (REF)

Cadmium accumulates primarily in producer plants from where it is transported to different consumer levels; from herbivores to top carnivores or ominivores. Certain plant species tends to over-accumulate HMs to protect them from herbivory. Cadmium accumulation in different trophic level is studied in various crop plants () and have been shown to trigger toxic effects on the members of food chains of the food web. This reduces the stable structure of soil, terrestrial and aquatic ecosystems. Excess Cd in soil has negative impacts on soil microbial ecosystem decreasing and/or altering their population to reduce soil composition, nature and fertility.

Toxicity of Cd is a major growth limiting factor for plants (Mohan and Hosetti, 2006) which reduces the quantity and quality of crop plants, and poses threat to environmental sustainability. In fresh water ecosystem, aquatic plants accumulates Cd, transfer them to sea foods including fishes and prawns. Cadmium is toxic for land plants and human beings (Shukla et al., 2007) and poses serious threat to safe food production. It mainly gets accumulated in human kidney and increases the risk of pulmonary emphysema and renal tubular damage (Godt et al., 2006; Bernard, 2008). Extreme cases of chronic Cd toxicity can result in osteomalacia, bone fractures, Itai-

Itai disease, anemia mainly in women over forty and induce hormesis (low dose stimulation and high dose inhibition) in plants (Calabrese and Baldwin, 2004).

2.2 Effect of cadmium on plant growth responses

2.2.1 Morphological effects

Cadmium interrupts various physiological processes which results into decreased growth attributes. Several workers have observed the Cd-induced visible affects the morphology of plants even at its low level (Van Assche and Clijisters, 1990). Visual symptoms, like reddish brown spots on primary and secondary leaves and as blackening, browning, burning and weakening of stems and leaves have been documented in higher plants, especially those of agricultural importance such as legumes and cereals (Anjum et al., 2008; Farooqi et al., 2009). The leaf margins appear reddish brown in color on veins, leaf becomes chlorotic under Cd toxicity (Faizan et al., 2012). It retards the growth of legumes such as *Glycine max* (Dewdy and Ham, 1997), *Pisum sativum* (Sandalio et al., 2001), *Medicago sativa* (Drazic et al., 2006), *Cicer aeritinum* (Hasan et al., 2008, Faizan et al., 2011) and other also crops including *Corchorus olitorius* (Mazen, 2004), *Zea mays* (Krantev et al., 2008), *Lycopersicon esculentum* (Çanakci et al., 2012, Hayat e al., 2013) and *Oryza sativa* (Uraguchi and Fujiwara, 2012). The rhizobial soil population and its interaction with roots are very sensitive to HM stress which ultimately reduced the dry weight of legumes (Rana and Ahmad, 2002; Khan et al., 2012). Cadmium retards seed germination, plant length, fresh and dry weight, leaf area per plant and number of nodules of legumes when given through soil (Hayat, 2010; Faizan et al., 2011; Irfan, 2014).

2.2.2 Plant anatomical changes

Ahmad et al., (2005) observed significant decrease in the size of stomata, stomatal pore, and their density on both upper and lower epidermis; length of trichomes increased while their density decreased. In the stem, cortex increased but proportion of pith and vasculature decreased, however, in the root the reverse condition occurs while density of vessel elements and xylem fiber in the wood of both stem and root decreased in *Trigonella foenum-graecum* at pre-flowering, flowering and post flowering stages under Cd and Pb stress. Cadmium with or without Cu reduced stomatal frequency, size of abaxial epidermal cells of leaflets, stomatal guard cells,

parenchyma of seedlings and metaxylem vessels in broad bean (Kasim, 2005). Cadmium negatively effects hormonal imbalance and water movement by reducing the size and number of xylem vessels (Poschenrieder and Barcelo, 1999).

2.2.3 Effects on physiological and biochemical responses

2.2.3.1 Effect on membrane stability

Binding of HMs to apoplastic membrane dislocates electrons from carrier proteins which results into a burst of reactive oxygen species (ROS). Low apoplastic antioxidant enzymes pool and activity of alternative oxidases further accelerate the generation of free radicals. Activated oxygen radicals like hydrogen peroxide, hydroxyl and superoxide radicals mediate membrane lipid peroxidation, oxidation of ion channels, transporter proteins and formation of secondary radicals. Membrane destabilization and disintegration cause disturbed osmotic balance across the plasma membrane. Cadmium-induced lipid peroxidation (Sandalio et al., 2001; Astolfi et al., 2004; Chaoui et al., 2004; Srivastava et al., 2004) and enhanced lipoxygenase activity is reported in the leaves (Sandalio et al., 2001; Khan et al., 2002; Panda and Khan, 2003). Excess level of Cd causes oxidative injuries such as lipid peroxidation which leads to alteration in membrane functioning, fatty acid composition and protein carbonylation (Romero-Puertas et al., 2004; Dalcorso et al., 2008; Popova et al., 2009) which is determined by the estimation of the content of thiobarbituric acid reactive substances (TBARS) or malondialdehyde (MDA) level as observed in *Brassica napus* (Doltani et al., 2006), mung bean (Yusuf et al., 2012) and chickpea seedlings (Faizan et al., 2011). The varietal difference in lipid peroxidation level and membrane stability index was also recorded in the leaves of *Vicia faba* (Yusuf et al., 2012), *Brassica juncea* (Irfan, 2014), *Lycopersicon esculentum* (Hasan, 2009) and *Cicer aerietinum* (Faizan et al., 2011).

2.2.3.2 Water uptake and turgor responses

The progressive buildup of metal content in plants concomitantly decreases growth, biochemical attributes and water content (Gonzalez et al., 2012). Roots and old leaves are the main sinks which offer a defense or tolerance mechanism to the plants to avoid their toxic levels. Entry of Cd through irrigated water could have detrimental effects on plant growth due to loss of water and nutrients. Marshner (2012) has suggested that increasing Cd concentration in leaves decreased the turgor pressure and increased

stomatal resistance which may be inferred due to increased ABA content (Poschenrieder et al., 1989; Hsu and Kao, 2004). Singh and Tiwari, (2003) measured the water status of *Brassica juncea*, and denoted that the suppression of growth at higher Cd level was due to water stress. Decreased germination of seeds and growth of cowpea seedlings grown in soil amended with various levels of Cd may be attributed to be the negative effects of Cd on water uptake and water movement (Vijayaragavan et al., 2011; Mondal et al., 2013).

2.2.3.3 Photosynthetic pigment compositions

Photosynthesis is the most important process of carbon fixation on this earth at primary level and it is regulated at several steps. Level and activity of photosynthetic pigments, availability of substrate, CO_2, water, and organization of thylakoid lamellae are important for this process. Cadmium accumulates preferably in chloroplast and thus disrupts its function (Bi et al., 2009) and disturbs membrane organization of thylakoids and chloroplast ultrastructure (Hakmaoui et al., 2007). Cadmium-mediated excess generation of ROS may induce degradation of chlorophyll (Padmaja et al., 1990). Cadmium may induce lipoxygenase activity which causes chlorophyll oxidation (Somashekaraiah et al., 1992). Decline in chlorophyll biosynthesis may partially be due to Cd-induced inhibition of enzymes needed for chlorophyll biosynthesis (Krupa and Baszynski, 1995; Muneer et al., 2011). Cadmium-mediated repression of chlorophyll level was also reported by Abdel Basset et al., (1995) through the induction of chlorophyll catabolizing enzyme i.e. chlorophyllase and cab1 (chlorophyll a/b binding protein1). It inactivates membrane light harvesting complex (LHC) of chloroplast, protein, proton pumps, and enzymes of photosynthesis to inhibit photosynthetic efficiency (Siedlecka and Baszynsky, 1993; Siedlecka and Krupa, 1996; Vassilev et al., 2005). Cadmium stress in root inhibits water as well as mineral uptake, transpiration rate and stomatal conductance as reported in soybean, clover and lucrene (Poschenrieder et al., 1989; Nazar et al. 2012). Reduced uptake of mineral ions such as Mg, Fe, Zn, Cl, N, P and K has negative effect on the biosynthesis of chlorophyll and other pigments (Poschenreider et al., 1989; Alcantara et al., 1994).

Carotenoid is unoxygenated compound and have long unsaturated aliphatic chains. Oxidative stress level its increase because it acts as an antioxidant molecule. One of the diverted products of the biosynthetic pathway of carotenoid is abscissic

acid. Cadmium-induced decrease in carotenoid may be due to its cleavage by environmental stress. Increase in ABA synthesis and senescence in sensitive legumes may be due to decline in carotenoid biosynthesis. Many authors reported the decrease in carotenoid content in *Brassica napus* (Larsen et al., 1998), *Vicia faba* (Kasim, 2005), *Vigna mungo* (Singh et al., 2008) and in *Daucus carota* and *Solanum tuberosum* (Flemotomou et al., 2011). Decreased photosynthetic performance due to Cd may be partially due to disorganization in the composition of photosystem I and II. The cumulative effect of Cd stress on different pigments resulted into decreased efficiency of photosynthesis in *Cajanus cajan* (Sheoran et al., 1990), *Raphanus sativus* (Krupa et al., 1993), *Pisum sativum* (Chugh and Sawhney, 1999), *Solanum lycopersum* (Dong et al., 2005), *Glycine max* (Shamsi et al., 2007), *Brassica juncea* (Hayat et al., 2007) and *Zea mays* (Ekmekci et al., 2008).

2.2.3.4 Mineral absorption

Cadmium interferes with the uptake and transport of nutrients through the roots (Quariti et al., 1997; Sanita'di Toppi and Gabbrielli, 1999; Zornoza et al., 2002; Carpena et al., 2003; Benavides et al., 2005; Dalcorso et al., 2008; Gozübenli, 2010) and thus compete for the active sites of different cations present in enzymes. It is also reported to inhibit the uptake of nutrient elements e.g. P, K, S, Ca, Zn, Mn and B in peas in an organ-type and genotype-dependent manner (Metwally et al., 2005). Cadmium restricts the availability of several micronutrients such as Fe, Ni, Cu (Baccouch et al., 1998; Siedlecka and Krupa, 1999) through plasma membrane, leading to deficiency in essential metal ions on growth and metabolism.

Cadmium hinders the essential macronutrients such as N, P and K (Hernandez et al., 1996; Table 4). It also disrupts *Rhizobium*-root signaling and thus affects nodule formation inhibit P uptake and its utilization for development of nodule (Lukiwatid and Simanungkalit, 2002; Gull et al., 2003). High Cd level induces early senescence in root nodules. It also decreases the population of plant growth promoting bacteria which enriches the soil with essential nutrients (Zaidi et al., 2003; Zaidi and Khan, 2006). Decreased uptake and availability of N, P and K have been reported in several plants under Cd stress (Ciecko et al., 2004) due to reduced microbial activity and symbiotic nitrogen fixation (Balestrasse et al., 2005; 2006; Pereira et al., 2006; Younis, 2007; Wei and Ma, 2010; Chaer et al., 2011, Hao et al., 2014; Murtaza et al.,

2014), root mineral transportation (Geneva et al., 2006; Hussain et al., 2012) and decline in transpiration rate (Kaznina et al., 2011).

2.2.3.5 Activity of primary metabolic enzymes

The decrease in symbiotic N_2 fixation and decreased microbial activity causes decline in N compounds which in turn decreases protein biosynthesis and its level in leaves (Younis, 2007; Krantev et al., 2008; Weisany et al., 2013) and in grains (Salgare and Acharekar, 1992; Hasan et al., 2008). Cadmium inhibits enzymes, proteins of membranes and insoluble fraction of cytoplasm. It has numerous negative effects on plant cells such as membrane distortion, activity of several group of enzymes such as enzymes of calvin cycle (Sandalio et al., 2001), N metabolism (Boussama et al., 1999), sugar metabolism (Verma and Dubey, 2001), sulphate assimilation (Lee and Leustek, 1999) and it also accelerate in leaf senescence (Siedlecka and Krupa, 1999). Germinating seedlings of pigeon pea when exposed to Cd altered the enzyme activity and thus mobilization of food reserves (Bishnoi et al., 1993). Carbonic anhydrase (CA) and nitrate reductase (NR) are important primary metabolic enzymes of carbon and N metabolism respectively. The decline in the activity of CA decreases CO_2 fixation and hence the activity of RuBPCase (Price et al., 1994; Stemler, 1998a; Majeau and Coleman, 1994), which might be brought about by Cd mediated decline in stomatal activity (Poschenrieder et al., 1989; Nazar et al. 2012).

Carbonic anhydrase activity decreased with the increase in the level of Cd in soil dose dependent manner. The higher level of Cd accumulation inhibits CA activity of leaf, whereas low Cd level stimulated its activity (Siedlecka and Krupa, 1996; Hasan et al., 2007; Khan et al., 2008). Higher level of Cd in soil antagonizes Zn (Costa and Morel, 1994), an important co-factor of CA enzyme (Escudero-Almanza et al., 2012). Cadmium-induced changes in CA are also reflected at the level of photosynthesis (Khan et al., 2008), as CA is assumed to be involved in photosynthetic electron transport system (Stemler, 1997) and maintains pH of the chloroplast during rapid changes in light intensity (Reed and Graham, 1981).

Inert N available in atmosphere is fixed to ammonia and then to nitrite and further into nitrate through biological N_2 fixation by *Rhizobium*. Nitrate reductase, the primary and rate determining step in the reduction of nitrate that is further assimilated to organic molecules which become building blocks for plant growth and

development (Solomonson and Barber, 1990). At the cellular level, NR activity is influenced by a series of environmental factors (Murphy et al., 1997). Decline of NR activity was observed in *Hydrilla verticillata* with the increasing Cd concentration, (Garg et al., 1997). A retarded assimilation of NO_3^- in the presence of soil Cd was reported in several plants (Hernandez et al., 1996; Quariti et al., 1997; Gouia et al., 2003; Hasan et al., 2008).

2.2.3.6 Generation of reactive radicals and oxidative stress

Cadmium induces production of toxic metabolites and ROS such as superoxide ($O_2^{\cdot-}$), hydrogen peroxide (H_2O_2), hydroxyl radicals ($^{\cdot}OH$) and singlet oxygen (O_2^{1}). This results in the inhibition of growth and photosynthesis (Shamsi et al., 2008). The plants have evolved defense mechanisms for mitigating these radicals in order to survive in Cd stress and repair the ROS-induced damages (Overmyer et al., 2003). These are specific but complex mechanisms involving morphological, physiological and biochemical adaptations. In particular, the ROS-combating antioxidant system consists of enzymatic antioxidants such as superoxide dismutase (SOD), peroxidase (POD), catalase (CAT), ascorbate peroxidase (APX) and glutathione reductase (GR), and non-enzymatic antioxidants which consist of glutathione (GSH) and ascorbic acid (Gill and Tuteja, 2010). Plants resistance to environmental stresses is dependent on the nature and the amount of antioxidants. Reactive oxygen radicals at supra optimal levels start stress signal at the cost of reduction of growth. Decline of growth, chlorophyll content, photosynthesis and stomatal conductance along with enhanced MDA level, SOD and POD activities were found in soybean plants exposed to Cd stress (Shamsi et al., 2008). Damodharam et al., (2009) reported significant inhibition in the activity of POD, CAT along with nitrate reductase (NR) and nitrite- reductase (NiR) in *Arachis hypogea* seedlings under Cd stress leading to decrease in the plant growth.

2.2.3.7 Antioxidant activity

The redox status of cell is regulated through the balance of oxidative level and antioxidant system activities. The antioxidant system consists of a number of enzymes and molecules that protects the cell organelles from oxidative damage (Smeets et al., 2005; Pal et al., 2006). Cadmium induces the oxidative stress (Hendy et al., 1992; Somashekaraiah et al., 1992) and stimulates the supraoptimal generation of free

oxygen radicals (Balakhnina et al., 2005; Demirevska-Kepova et al., 2006). Enzymatic scavengers of activated oxygen are POX, CAT and SOD (Sandalio et al., 2001; Khan et al., 2002; Bor et al., 2003; Panda and Khan, 2003; Chaoui et al., 2004; Demiral and Turkan, 2005; Mandhania et al., 2006).

Superoxide dismutase quenches the superoxides and hence it is considered as first line of defense (Polle and Rennenberg, 1994). Cadmium induces the activity of SOD in mustard (Hayat et al., 2007), cucumber (Goncalves et al., 2007) and in maize (Ekmekci et al., 2008) may decrease the level of enzymatic or nonenzymatic antioxidants (Sandalio et al., 2001; Balestrasse et al., 2001; Fornazier et al., 2002; Cho and Seo, 2005; Mohan and Hosetti, 2006).

Cadmium-induced activity of POX was reported in legumes plants as soybean (Fuhrer, 1982), bean leaves (Lee et al., 1996), chickpea (Hasan et al., 2008), and other plants as in roots leaves of rice (Reddy and Prasad, 1993), mustard (Singh and Tiwari, 2003; Hayat et al., 2007), Indian pennywort (Mishra et al., 2006), leaves of cucumber (Goncalves et al., 2007), *Calamus tenuis* (Khan and Patra, 2007) and maize (Ekmekci et al., 2008). Catalase is another class of enzymes activated by oxidative stress which converts H_2O_2 to H_2O and O_2. The CAT activity was decreased while the activities of guaiacol peroxidase (GPX) and ascorbate peroxidase (APX) were increased in *Phaseolus aureus* on exposure to Cd (Shaw, 1995; John et al., 2007).

Cadmium stress increased the activity of CAT in wheat (Milone et al., 2003), rice (Panda and Patra, 2007) and chickpea (Hasan et al., 2008). No affects of Cd treatment was observed in maize plants (Krantev et al., 2008). Antioxidant molecules include ascorbate, glutathione, tocopherol and carotenoids etc. which may work in association with these antioxidant enzymes. In addition to these antioxidant molecules, low molecular weight thiol buffers also possess strong antioxidant properties which could counteract Cd-induced oxidative stress (Pichorner et al., 1993; Shanthala et al., 2006).

2.2.3.8 Plant provisions to withstand excess heavy metals

Primary strategy in most of the plants especially sensitive plants to avoid the metal uptake is through various mechanisms such as redirection of root growth, root hair regulation, increased accumulation of HMs in neighboring crops to legumes (Liu et al., 2012; 2013) or metal efflux. Dilution of metal at the community level is another

strategy where mixed populations have mild effect of excess soil HMs on sensitive plants and thus enable them to survive (Rather, 2013). Therefore, plants growing in such areas are naturally selected to study their defense mechanisms to counter toxicity of metals. Still excess metals available in soil are inevitable and are readily absorbed through root system to plant tissues. Therefore, different plants have different internal resistance to absorb different levels of HM. On the basis of which they are classified as metal-sensitive, moderate or tolerant plants. The activation of defense is a costly affair and channelizes the growth metabolisms, hence, plants often compromise growth, get threatened or could be completely eliminated from the community (Rather, 2013).

The majority of research has focussed on physiological mechanisms of metal uptake, transport and sequestration but little is known about the genetic basis of hyperaccumulation. The genetic basis of Cd tolerance and hyperaccumulation was investigated in *Arabidopsis halleri*, which showed that Cd tolerance might be governed by more than one major gene (Bert et al., 2003). The mechanism of Cd tolerance and hyperaccumulation in *Thalaspi caerulescens* is probably due to the presence of hairy roots which withstands the effects of plasma membrane depolarization (Boominathan and Doran, 2003). There is known genetic polymorphism among different plant and species by which some are capable of hyperaccumulation while others are not. This is in contrast to the phenomenon of metal tolerance. Plant species which possess metal tolerance are polymorphic and evolve tolerance only in local population on metalliferous soils (Pollards et al., 2002). Plants are often screened against locally prevailing different stress factors. A distinct group of plants have shown to tolerate HM concentrations which are called hyperaccumulators and are capable of sequestering metals in their tissues at a high concentration which would be toxic to most of the organism. The plants species rich in sulphur compounds or other chelating agents could reach to such a limit that they hyperaccumulate these HMs (Na and Salt, 2011). These crops can potentially be used or optimized in phytoremediation programs for HM detoxification of polluted sites and also for phytomining, which is considered as a safe and natural alternative of other strategies of metal decontamination of such sites. Agricultural crops are avoided for such programs. Although some degree of hyperaccumulation occurs in all the members of the species that can hyperaccumulate but there is always genetic

variations in the ability of the plants to hyperaccumulate both between and within the population (Pollards et al., 2002).

2.3 Benefits of microbial association to leguminous plants

The successful germination of seed and its subsequent growth into plant is determined by the prevailing conditions of its habitat. The plant community in vicinity and interaction of rhizospheric microbial population influence these factors (Mar Vazquez et al., 2000; Soderberg et al., 2002; Liu et al, 2012; Rather, 2013). Arbuscular mycorrhizal fungi regulate plant growth, nutrient acquisition, yield and increase soil fertility (Barea, 2000; 2002). Phosphate solubilizing rhizobacteria, *Pseudomonas* and symbiotic rhizobial strains which positively regulate plant growth under natural (Smith et al., 1997; Lesueur et al., 2005) and stressful conditions which may be biotic such as soil pathogens (Azcon-Aguilar et al., 1996; Dar et al., 1997; Demir et al., 2007; Aysan and Demir, 2009) or abiotic such as drought (Ruiz-Lozano et al., 2001; Porcel et al., 2006; Franzini et al., 2013), nutrition source and HM stress etc. (Wani et al., 2007c, 2008a,b). Cadmium-induced toxicity symptoms are multiple such as desiccation, decline in soil-microbial activity, nutritional deficiency and damage of plant. The root association of mycorrhizae or *Rhizobium* is important for legumes to manage soil mineral acquisition and growth (Mortimer et al., 2008; Azcon and Barea, 2010) which is negatively regulated by high level of metal in soil (Zaidi et al., 2012). However, metal resistant population of AM fungi or rhizobia has been isolated and evolved for better performance (Tobar et al., 1996; Routary et al., 1997; Raizada et al., 1998; Wani et al., 2007d,e). Dual inoculated legumes with mycorrhizal fungi and *Rhizobium* assess the improvement of their nutritional efficiency and this was worked out by several workers (Marques et al., 2001; Barea et al., 2002; Aryal et al., 2003; Jia et al., 2004; Antunes et al., 2006a,b; Zaidi et al., 2007; Wu et al., 2009), which is shown to have increased plant biomass and seed yield (Abusuwar and Ahmed, 2003; Xavier et al., 2003; Galal et al., 2003). Arbuscular mycorrhizal fungi also increase the nitrogenase, nitrate reductase, phosphatase activity and the level of cytokinins in legumes (Goicoechea et al., 1996; Foo et al., 2013) as well as phosphatase activity in non-legumes (Rubioa et al., 1990) to enrich the plant metabolism. Arbuscular mycorrhizal fungi increase the uptake of P and N and thus induce growth and metabolism of plants (Abusuwar et al., 1997; Galal et al., 2003; Faizan et al., 2004) (Table 3).

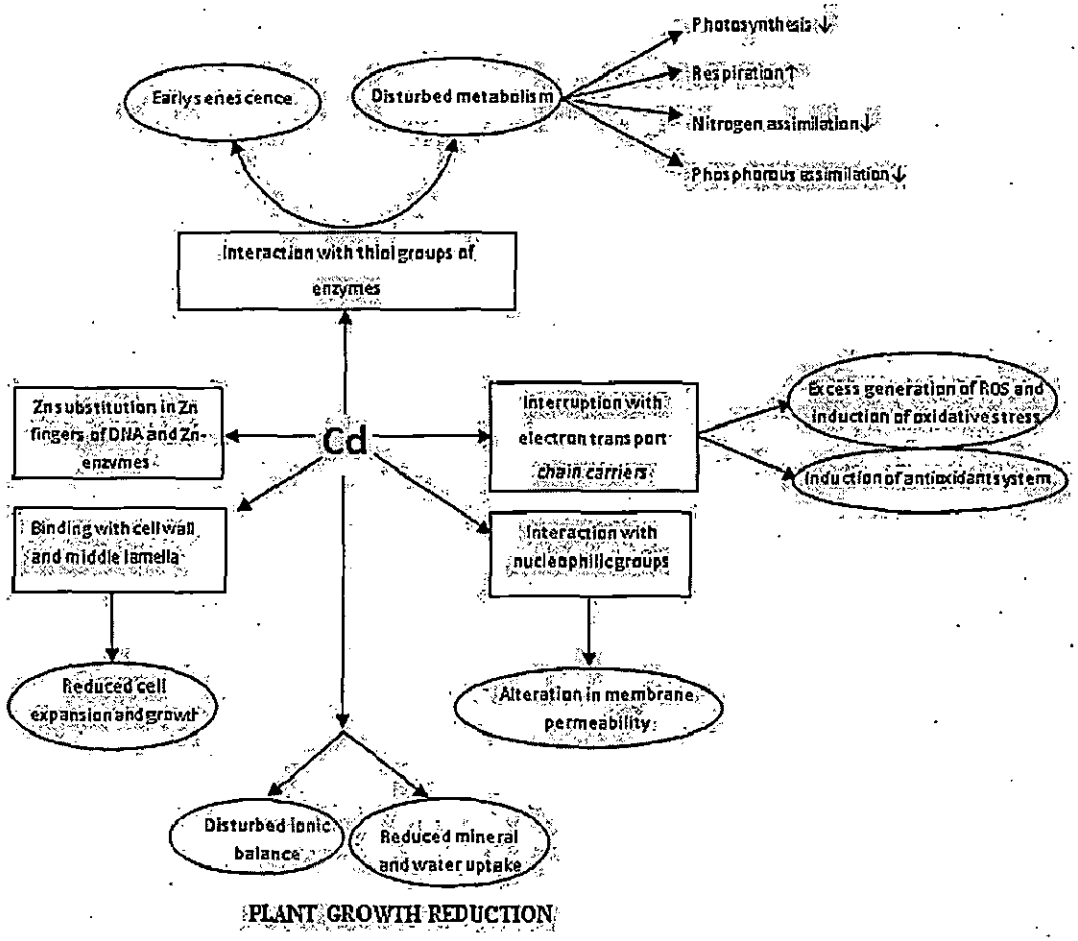


Figure 2.2: Cadmium induced toxicity processes at cellular level.

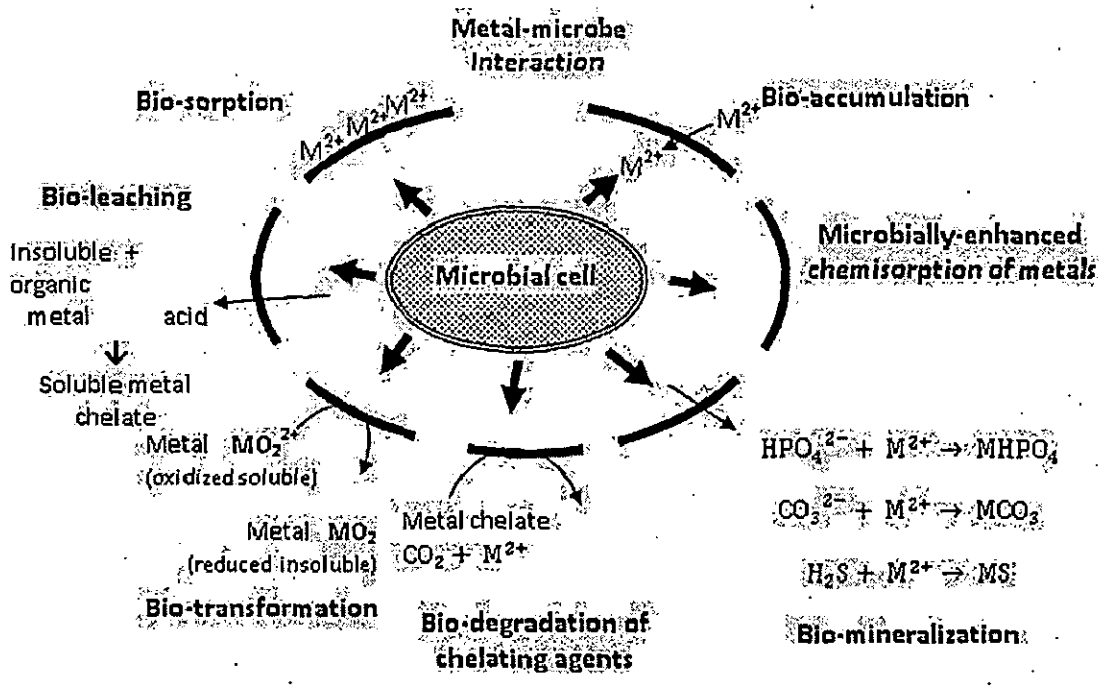


Figure 2.4: Microorganism counteract toxic level heavy metals deploying many mechanisms (modified from Khan et al., 2009).

Table 2.3: Ways of heavy metals and mechanism (including Cd) rhizo-remediation and legume growth improvement in tripartite association

A.	***Rhizobium associated metal rhizo-remediation***
1.	Strengthening plant immunity to combat metal stress by providing N_2-economy
2.	Heavy metal dilution by incorporating them in *Rhizobium* metabolism
3.	Bio-sorption of heavy metals in cell wall, other structural components e.g. pigments; or as enzyme co-factors
4.	Plant growth induction through the secretion of phytohormones
B.	***Mycorrhiza mediated metal detoxification rhizo-remediation***
1.	Sieving of heavy meals into roots of legumes
2.	Detoxification of heavy metals through the secretion of organic acids, metallo-thionines etc.
3.	Nutritional supplementation to strengthen plants internal resistance viz. phosphates, mineral elements and soil organic compounds
4.	Enrichment of rhizosphere with PGPRs
5.	Plant growth improvement through secretion of phytohormones
6.	Protection from root disease causing pathogens
7.	Protection from water stress
8.	Incorporation of heavy metals in fungal metabolism (metal sink)
9.	Protection of *Rhizobium*-legume symbiosis
C.	***Plant strategies and defense mechanisms to overcome metal stress***
1.	Direction of root growth and morphology
2.	Retention of metal in root
3.	Transportation to metabolically inactive parts
4.	Cellular detoxification and sequestration to vacuoles
5.	Secretion of phyto-chelatins (PCs) and metalothionines (MTs)
6.	Antioxidant enzymes (CAT, POX, SOD, GR etc.) & molecules (glutathione, ascorbate etc.)
7.	Reactive oxygen species quenching metabolites and osmo-protectants (proline, polyamines, betains and sugar alcohols)
8.	Excretion of organic acids by root
9.	Efflux of heavy metals activating metal exporters

2.4 Cadmium toxicity on soil rhizobial population and nodule metabolism

Soil HM contamination causes significant alteration in the population and activity of soil microbes (Paudyal et al., 2007; Wani et al., 2008a, b; Khan et al., 2009b; Krujatz

et al., 2011) and reduce soil fertility, this indirectly deplete the nutrient pool, uptake of nutrient and ultimately affects plant health (Karpiscak et al., 2001). Adverse effects of metal enriched soil have been observed in several legumes (Wani et al., 2007a, b, 2008a, 2010). In legumes, the N demand is fulfilled through symbiotic N_2 fixation wherein atmospheric N_2 is converted to usable form of N i.e. NH_3 by nitrogenase enzymes present in nodules (Shiferaw et al., 2004). Rhizobial Nod factors trigger the mitotic cell division in roots, leading finally to nodule formation (Matiru and Dakora, 2004; Jones et al., 2007; Batut et al., 2011). The direct effect of HMs has been reported to limit the growth of *Rhizobium* and host legumes (Heckman, et al., 1987; Broos et al., 2005). In some legumes, excess HMs delay the nodulation process (Reichman, 2007). The decreasing effect of Cd on plant roots inoculated with sensitive and resistant strain reflected the difference of nearly 27% on nodulation and nitrogen level of *Sinorhizobium meliloti* (Sepehri et al., 2006). Heavy metal contamination has shown inhibition of N_2 fixation by the strain *of Rhizobium leguminosarum* in *Trifolium repens* (Hirsch et al., 1993) and in faba bean (Chaudri et al., 2000). The reduction in the activity of nitrogenase in field and pot trials with decreased nodulation, N metabolism and plant growth was also reported (Ahmad et al., 2012). Heavy metals at toxic level also interfere the induction of root hairs, mineral absorption, normal metabolism and growth morphology. The interaction of *Rhizobium* in the nodules of chickpea was found to be very sensitive to HMs resulting in a decrease of dry mass of chickpea and green gram (Rana and Ahmad, 2002). Faizan et al., (2011) reported that the application of Cd to enhanced the seedling mortality of six cultivars of chickpea. Higher concentrations of HMs severely damaged the metabolic activities of legumes such as soyabean, pea, *Medicago sativa*, and chick pea (Sandalio et al., 2001; Drazic et al., 2006; Hasan et al., 2008). Nonessential HMs such as Cd are even more toxic to plants at lower concentrations (Bahmani et al., 2012). It competes with the uptake of other minerals and thus cause their deficiency (Gadd, 2007, 2010), induce oxidative stress, inhibits enzyme activities (Noriega et al., 2007; Irfan, 2014), and alter membrane functions and net photosynthesis (Pandey and Tripathi, 2011; Chen et al., 2011; Irfan et al., 2013). The damaging impacts of excessive uptake of Cd on plant growth was marked in various plant species viz. *Glycine max* (Dewdy and Ham, 1997), *Pisum sativum* (Sandalio et al., 2001), *Medicago sativa* (Drazic et al., 2006), *Vigna radiata* (Wahid et al., 2007) and *Cicer arietinum* (Hasan et al., 2008).

2.5 Strategy of legumes to overcome heavy metal stress

On the other hand, pulses have been found to redirect the Cd accumulation in the neighboring crops (Liu et al., 2012; 2013). Excess HMs adversely influence population of soil microorganisms, affecting their growth, abundance, genetic diversity, nodulation ability, and symbiotic efficacy under different doses of availabity and also with cooperation (Ahmad et al. 2012). The tolerance or avoidance to excess soil HM could be increased by the association of plant roots with several plant growth promoting microorganisms (PGPMs). These could potentially sieve metals at the interface of root and soil or root to plant, reducing the metal transport to shoots. Arbuscular mycorrhizal fungi, phosphate solubilizing bacteria (PSB), root noodling rhizobia etc. are the good example of rhizofilteration of toxic level of HMs (Ganesan, 2012). Plants secrete both high and low-molecular weight compounds from their roots, and these root exudates function not only as nutrients for soil microbes but as signal molecules in plant-microbe interactions. Leguminous plants establish symbiotic interactions with rhizobia and AM fungi to obtain several nutrients such as N and P. In these interactions, flavonoids and strigolactones in root exudates serve as signal molecules to establish the symbiotic interactions. Root exudates from some legume plants also function to acidify surrounding soils to acquire phosphate (Sugiyama and Yazaki, 2012). Inoculation of AM fungi with rhizobia strain further detoxifies the metal in soil and discourages its uptake. However, it is not possible for a plant growing in HM polluted area to completely avoid the toxic level of essential or non-essential HM.

2.5.1 Role of AM fungi in prevention of cadmium toxicity in legumes

The importance of legumes has been attributed to the determination of N economy of various ecosystems (Makoi et al., 2009) including arable lands by forming root nodules (Ma et al., 2006). Nonessential HMs and excess of essential HMs have strong negative correlation with plant growth (Vyas and Puranik, 1993; Shetty et al., 1994; Emamverdian et al., 2015). Cadmium, being nonessential metal inhibits nodule formation and N-fixation in legumes (Hernandez et al., 1995), decrease nutrient uptake (Obata and Umebayashi, 1997), photosynthetic activity (Kumar and Kumar, 1999) and finally biomass production (Leita et al., 1993). The deleterious effect of HMs taken up by soil environment could be minimized by the use of *Rhizobium* (Khan et al., 2009a, c; Kumar, 2012) and mycorrhiza (Heggo et al., 1990; Saraswat

and Rai, 2011) which are called as agents of rhizoremediation (Kuiper et al., 2004). Soil microbial pool detoxifies HMs like Cd, Hg and Pb (Aiking et al., 1985). Plant root-mycorrhizhal symbiosis is one of the important associations amongst plant microbe interaction. It is so important that over 95% of the plant families are known to have mycorrhizal association under normal conditions. The use of mycorrhizae could potentially minimize the fertilization and water. It aids nutritional supplementation to the host plant at the cost of carbohydrate. The nutritional availability which on one hand strengthens plant immunity against abiotic and biotic stresses, on the other hand fungus itself protects plants from root pathogens, metal toxicity and water stress. Role of mineral nutrition in minimizing Cd accumulation by plants was reported by Sarwar et al., (2010) in agricultural fields. Amongst different flowering plants which associates AM fungi with them, legumes are of special importance. Legumes substantially contribute to the soil N pool and productivity in terrestrial ecosystems (Cleveland et al., 1999). It has also been observed that AM fungus, when associates themselves in the tripartite relationship with *Rhizobium*-legume roots, strengthens the nodulation frequency and N fixation efficiency of host plant. Since HMs persists in soil for a long time they are very resistant to chemical degradation or physical removal or immobilization (Kroopnick, 1994). The site specific management, excavation and disposal is cumbersome and uneconomic (Parker, 1994; Elliott et al., 1989). Bioremediation in this context is a better substitute (Leyval et al., 2002) which involves microbial contamination and climatic conduciveness (Brar et al., 2006). Researchers have shown that AM fungi inoculation significantly improves the tolerance of legumes to HM toxicity under different growing conditions (Chen et al., 2007). Mycorrhizae and rhizobia colonization besides protection of plant, also secrete phytohormones viz. cytokinins and gibberellins which induce cell division, stem elongation, seed germination and other functions of host plant. Positive benefits of composite inoculation of AM fungi and *Rhizobium* with legumes grown in metal contaminated soil are also reviewed by Muleta and Woyessa (2012). The diagrammatic representation of role of legume mycorrhization in preventing *Rhizobium* symbiosis and plant growth under HM stress has been shown in figure 2.3 various provisions and mechanisms of tripartite association in legume improvement are tabulated in Table 1.

Microbes themselves deploy several strategies to check metal uptake viz. cell wall biosorption, incorporation of enzymes and pigments (Gadd, 2009, 2010) and removal by metal efflux pumps and metal binding proteins and peptides (Silver, 1996). Low molecular weight organic acids such as oxalate and citrate secreted by roots to mobilize metals in soil. Synergistic interaction of AM fungi improves mobilization and detoxify these metals as fungi aided with plethora of diverse phytochelatins (PCs), metallothionines (MTs) and organic acids (Joner et al., 2000a, b) and easily change their strains as per requirement as compared to complex eukaryotes. Site specific optimization of AM fungi mediated mycorrhizal-remediation have been effective tool for restoring the economy of soil and plant (Takacs, 2012) particularly in legumes (Muleta and Woyessa, 2012). However, contradictory reports regarding increase or decrease of metal concentration of mycorrhizal plant are available (Tonin et al, 2001; Karimi et al., 2011). Enhanced uptake of HMs is the part of phytoremediation and reclamation of contaminated soil, whereas, HM are accumulated in fungal mat and thus uptake in checked (Rivera-Becerril, 2002; Jamal et al., 2002; Audet and Charest, 2007) in the plant tissue for safe food. Muleta, (2010) discussed the dependency of legumes on mycorrhizal associations. This association has shown to supply high P for nodulation and N_2-fixation as P is known as critical element for nodule formation (Barea and Azcon-Aguilar, 1983). The effect of dual inoculation of AMF and bacteria remarkably improve the HM tolerance of plant (Vivas et al., 2003a, b; Muleta, 2010). The mechanisms of HM tolerance by mycorrhizal legumes have been discussed by Malcova et al., (2003); Cardoso and Kuyper (2006), Garg and Aggarwal (2011) (Figure 2.4).

2.5.2 Benefits of tripartite relation for soil health

Evidence of existence of symbiosis of plant roots with AM fungus has been found in fossils dating back 460 million years ago. Mycorrhizal symbiosis predates the evolution of nodulation by approximately 400 million years. The sharing of two symbionts was shown to be present in cereals and essential for mycorrhizal signaling (Hayat et al., 2012). Arbuscular mycorrhizal fungus increases the absorption of N, P, K and S uptake besides the absorption of mineral elements e.g. Cu, Fe, Ni, Co and Zn. The fungal hyphae keeps check on the root uptake of excess level of Zn, Cd and Mn from soil. This protects the plant roots and enhances the ability of plants to revegetate and stabilize the soils of reclaimed mines that may be high in HMs. Increased stability

of *Rhizobium*- legume symbiosis ensures the fixation of optimum level of N_2 from air. The glycoprotein glomalin excreted by fungal hyphae act as glue which causes soil particles to stick together, or aggregate. Soil aggregates are resistant to breakdown by water and enhance physical characteristics of soil erosion by water and air. The fungal hyphae also physically entangle non-aggregated soil particles together, facilitating other bacterial and fungal compounds to form these particles into aggregates besides enriching rhizosphere with the activity of other beneficial microbes (Kumar, 2012). These aggregates are important for the soil food web.

Table 2.4: Effect of *Rhizobium* and/or AM fungi on heavy-metal/nutrient treated/non-treated (naturally growing) plants

Plant(s)	Treatment(s)	Effect(s)	References
Cadmium			
Tomato	Cd & Cu	Oxidative stress and antioxidant response	Chamseddine et al., 2009
Tomato	Cd	Accumulation of MDA and increase in the activity of SOD & POD with increased root, stem & leaf Cd level	Dong et al., 2006
Bean	Cd & Zu	Lipid peroxidation & antioxidant enzyme activities	Chaoui et al., 1997
Soybean		Nodulation and N_2-fixation	Chen et al., 2003
Soybean seedlings	Cd	Glutathione reductase activity increased in roots by Cd in hydroponic culture	Ferreira et al., 2002
Soybean	Cd	Macro-and micro-nutrient contents	Drazic et al., 2004
Thale cress	Cd	Antioxidant system, H_2O_2 content & lipid peroxidation	Cho & Sohn, 2004
Oats, maize, yellow lupine, radish	Cd	Plant yield & macro- & microelements content	Ciecko et al., 2004
Rapeseed	Cd	Photosynthetic pigments, sugars & MDA content	Doltani et al., 2006
Tobacco	Cd	Cd-resistant cell lines potential	Domazlika & Opatrny, 1989
Spartina densiflora	Cd	Oxidative stress and antioxidant defense response	Dominguez et al., 2010
Gracilaria domingensis	Cd	Growth, photosynthetic-performance, pigments, biochemical parameters, chloroplasts structure	dos Santos et al., 2012
Egg plant		Depression in growth, biomass and fruit yield of plants, catalase and ascorbate >peroxidase,< Fe and Zn > proline	Dube et al., 2009
Cowpea	Cd	Seed viability as per cent germination & shoot elongation rate decreased	Egharevba & Omoregie, 2010
Radish	Cd	Leaf area, chlorophyll (a, b), carotenoid decreased with increased root > shoot Cd distribution &	El-Beltagi et al., 2010

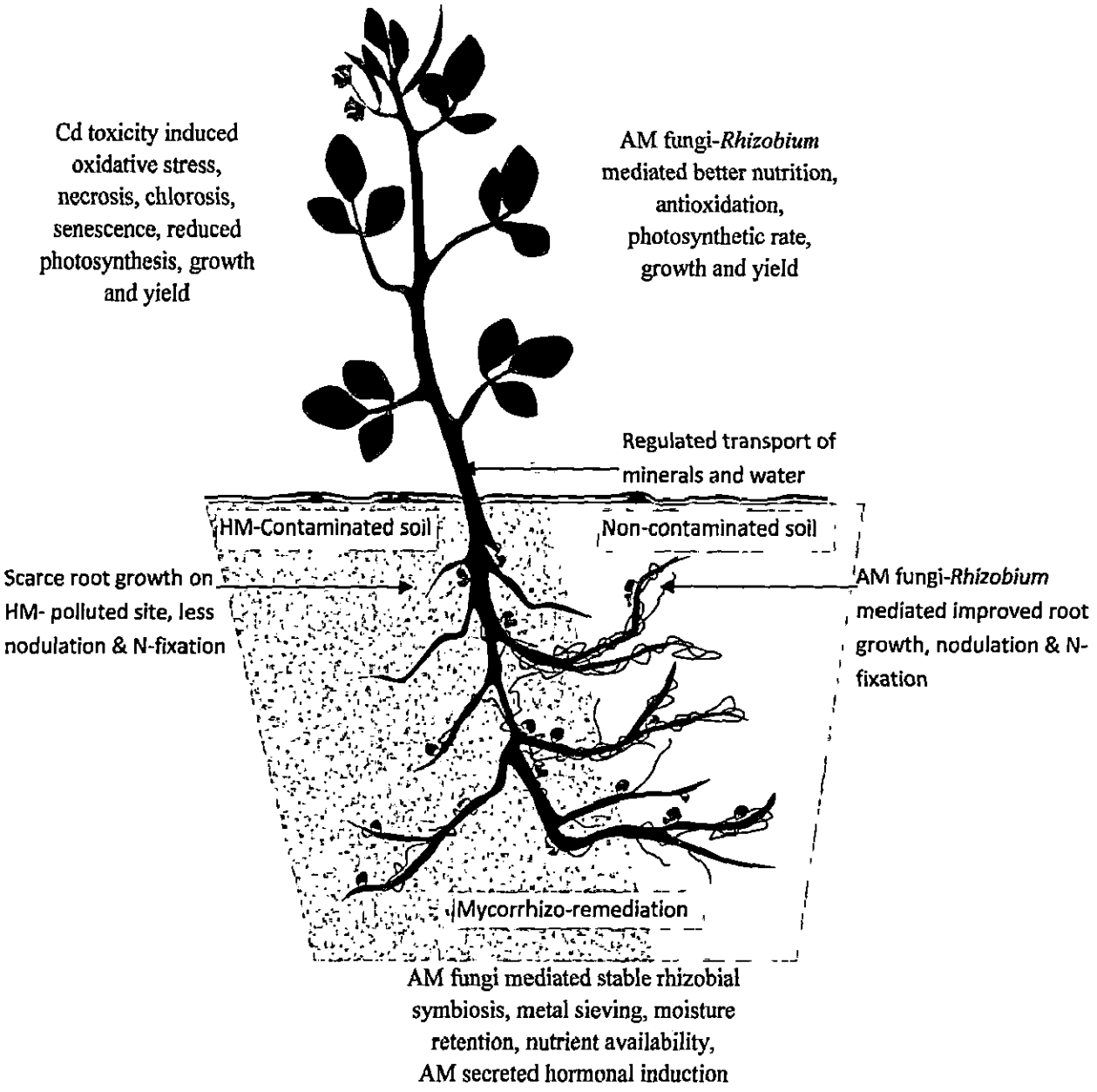


Figure 2.3: Role of AM fungi-*Rhizobium* mediated alleviation of Cd toxicity in legumes.

		antioxidant enzymes	
Almond	Cd	Reduced dry matter of leaves & roots, chlorophyll content, while sugar level & MDA level increased in leaves	Nada et al., 2007
Chickpea cultivars	Cd	Growth declined with physiological aspects (photosynthetic area, carbonic anhydrase activity & yield) while proline level increased	Faizan et al., 2012
Chickpea cultivars	Cd	Varietal differences in seedling mortality with visible Cd toxicity symptoms; chlorosis, necrosis etc.	Faizan et al., 2011
Albizia lebbeck	Pb & Cd	Toxicity (Cd > Pb) on germination & seedling growth	Farooqi et al., 2009
Sugarcane	Cd	Activity of CAT decreased while GR activity increased	Fornazier et al., 2002
***Rhizobium* strain(s)**			
White lupin	Natural	Altered nodule structure & function, decreased LegHb, amino acids, proteins, nutrient status as P, K, increased lipid peroxidation & thiols	Fornazier et al., 2002
Bean	Cd	Cd decreased water & nitrate uptake, activity of nitrate reductase (restricting Mo binding), Glutamate synthase. Cd increased the glutamate dehydrogenase activity with the level of ammonium	Gouia et al., 2000
Chickpea	Cr	Metal detoxification, improvement of growth by *Mesorhizobium* strain	Wani et al., 2008
Chick pea	Natural	Bacterized seeds showed increased length weight & seedlings germination	Nishita & Joshi, 2010
Faba bean	Cd	Cd (200 ppm) significantly reduced nodulation, nitrogenase activity, legHb and protein level, root & shoot dry matter	Elenany & Abd-Alla, 1995
Faba bean	Natural	Reduced glutathione (GSH) protected *Rhizobium leguminosarum* sensitive strain from oxidative damage	Corticeiro et al., 2006
Jack bean	Cd & P	AMF taken up the Cd from culture medium & restricted it in roots to transport shoots. AMF maintained lower peroxidase activity in roots.	de Andrade et al., 2005
Mung bean	N fertilization	Seed inoculation better enhanced yield over soil inoculation	Anjum et al., 200
Mash bean	P	Increased growth & yield but not seed protein	Murtaza et al., 2014
Yellow lupin	HM contaminated mine spill soil	*Bradyrhizobium* sp. 750 increased biomass, N content	Dary et al., 2010
Faba bean, Soybean, Lupin	Sewage sludge (Cu & Zn)	>30% application inhibited nodulation	Abd-Alla et al., 1991
Faba bean, Lentil	Cd, Pb, Zn,	Bacteria isolates from *Vicia faba*	Fatnassi et al., 20

Chickpea and *Sulla coronaria*	Cu rich soil	(*Rhizobium leguminosarum, R. etli, R. phaseolus & Agrobacterium*) were resistant to HMs than other legumes	
Rice	Cd	N availability increased the Cd tolerance of rice in genotype dependent manner	Du et al., 2009
Maize and lettuce	Low P	Growth promotion by phosphate solubilizing *Rhizobium leguminosarum biovar phaseoli*	Chabot et al., 1996
Sweet clover	Cd, As	Reduced nodulation with different Cd & As salts	Neumann et al., 1998
White clover	Cd, Pb, Zn	Reduction of nodule and plant size, and in nitrogenase activity in most heavily contaminated soil	Rother & Millbank, 1983
AM Fungi			
Medicago truncatula	Cd	Cd stress alleviation through modification in proteomic profile	Aloui et al., 2011
Faba bean	Cu, Zn, Pb, Cd	Mycorrhizal plants increased growth & tolerance in HM toxicity by increasing P uptake & decreasing HM transport, oxidative stress & DNA damage	Zhang et al., 2006
Red clover	Natural	Soluble protein content, SOD isozyme increased	Arines et al., 1993
Sunflower	Cd	Higher photosynthetic pigment, shoot P level, S, K, N, Fe Cu & Ca level	de Andrade et al., 2008
Citrus plant	P application	Nutrient status (P & K) increased with growth	Antunes & Cerdoso, 1991
Trifolium	HM multi-contaminated & lingo-cellulose agrowaste amended soil	Antioxidant activities and metal acquisition	Azcon et al., 2009
Lettuce	P & N	Nutrient acquisition increased	Azcon et al., 2003
Plantago major	Phosphate	Increased growth, shoot to root ratio, and P level more in mycorrhizal plant	Bass & Lambers, 1998
Sunflower, onion	Natural	Plasma membrane ATPase activity increased in sunflower root microsomes while in onion tonoplast, mitochondria membrane ATPase activity decreased	Bago et al., 1997
Lentil varieties	N & P	Increased nodulation and yield	Chowdhary et al., 1974
Wheat	Phosphorous	Soil available P status determines indigenous mycorrhizal colonization. With P fertilization, grain yield & shoot dry matter increased	Covacevich et al., 2007
Potato	Low P, high Al	Flavonoid and yield increased with mycorrhizal colonization	Davies et al., 2005
Tomato	Natural	Colonization patterns in a mycorrhiza-defective mutant plants	Gao et al., 2001

Pigeon pea	Cd & Pb	Higher mycorrhization & PC glutathione production in genotype dependent manner imparted improved growth, N_2 fixation in two varieties	Garg & Aggarwal, 2012
Pigeon pea	Cd & Pb	Increased antioxidant enzymes activity, AM fungi arrested Cd & Pb in roots, increased GR activity, decreased lipid peroxidation and electrolyte leakage.	Garg & Aggarwal, 2011
Pigeon pea	Cd stress	AM fungi improved N, P, Fe uptake decreased TBARS, H_2O_2 & nodule senescence, amended nodule functioning & antioxidant activity in genotype & concentration dependent manner.	Garg & Bhandari, 2012
Dual-inoculation			
Alfalfa cultivars	Natural	Increased density and seed yield of plant	Abusuwar and Ahmad, 2003
Alfalfa	Phosphorus	Increased seed yield, P & plant density	Abusuwar and Ahmad, 1997
Alfalfa	^{15}N, ^{32}P	N and P accumulation improved	Barea et al., 2002
Albizia lebbek & *Dalbergia sissoo*	Natural	Enhanced growth, biomass and nodule number	Nidhi & Rahangdale, 1999
Beans	Organic fertilizers	Improved growth, yield and nutrient uptake	Aryal et al., 2003
Bean	Phosphorus	Increased P stimulates nodule dry weight, activity and fungal colonies	Bethlenfalvay et al. 1982
Broad bean	N & P accumulation	increased N and P content influence biomass production, leaf area, net photosynthetic rate & % colonization	Jia et al., 2004
Carrot	Cys, Met, Glutathione	Root S acquisition through the AM symbiosis	Allen & Shachar-Hill, 2009
Cowpeas	Natural	Growth and chlorophyll content increased	Arumugam et al., 2010
Cowpea	Natural	Increased shoot & root length, dry weight, nodules dry weight, per cent mycorrhizal infection, chlorophylls levels	Arumugam et al., 2003
Chickpea	N & P	Enhanced root -AM colonization, root nodulation, plant growth & yield	Champavat, 1990
Chickpea	P deficient soil	Interactive effective synergistic, seed response protein, photosynthetic pigments & nodules number	Zaidi & Khan, 200
Chickpea	Natural	Dual inoculation enhanced the nodulation, N & P level, and yield	Subba Rao et al.,1986
Chickpea		Co-inoculation of N_2 fixing & P solubilizing bacteria promoted growth, nutrients & yield	Wani et al., 2007d
Centrolobium tomentosum	Natural	Increased plant height and dry weight	Marques et al., 200
Groundnut	P application	Increased nodule number, N content, root &shoot dry weight	Lekberg & Koide, 2005
Pigeon pea,	Phosphorus	Increased nodulation, mycorrhizal	Manjunath &

cowpea		colonization, dry weight, N & P content	Bagyaraj, 1984
Pea plants	Phosphorus	Increased growth, root AM colonization, nodulation, N_2 fixation, photosynthesis, acid phosphatase activity and P content	Geneva et al., 2006
Pea	Natural	Shoot dry matter production was significantly correlated with the total shoot N and P content. Co-selection of AMF species and *Rhizobium* strain enhanced pea nutrition & yield	Xavier & Germida, 2003
Soybeans	Phosphorus	Increase growth & yield, nodulation, improved P uptake	Asimi et al., 1980
Tobacco	Cd/other metals	Increased Cd immobilization in rhizo-spheric by AM extra radical mycelium	Janouskova et al., 2010
Wheat	N & P fertilizer	Dual inoculation increased shoot dry weight, nodules, N &P uptake and yield	Galal et al., 2003

2.6 Critical appraisal of review of literature

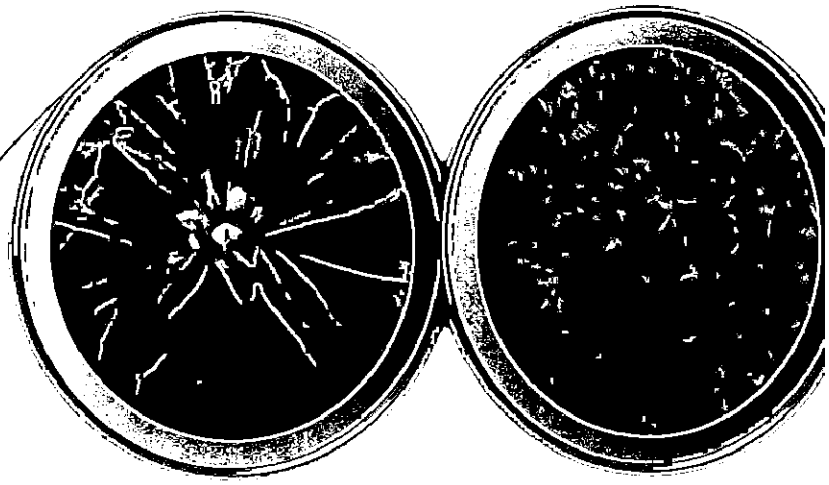
The literature reviewed above includes studies on the growth, productivity and physiological analysis of various crop plants under Cd stress. It appears that there are a few reports concerning the effect of application of symbiotic microbes in the rhizosphere (especially *Rhizobium* and Arbuscular mycorrhizal fungi) under abiotic stress on various physiological processes, including productivity of legumes, an important group of N fixing crops. Infact, the work on various crop plants is invariably aimed at establishing mechanisms of antioxidant machinery of plants exposed to various abiotic stresses. However, in-depth understanding of response of various microbes in enhancing and/or strengthening the antioxidant machinery to counteract the effects of abiotic stress-induced ROS production has not been done.

Only a few reports available in the reviewed literature confirmed the central role of *Rhizobium* and AM fungi in HM tolerance and amelioration of Cd toxicity but none of the concerted efforts by various research domains involve the aspect of enhancement of plant growth and reduced metal translocation, caused by the synergistic interaction of these symbionts in the microbial ecosystem in soil contaminated with Cd and still many key questions in the literature survey remain unanswered.

The reported study was undertaken on some selected leguminous crops to explore the possibility of ameliorating the adverse effects of Cd through the inoculation of *Rhizobium* and AM fungi. Following the application of these microbes to Cd-treated legumes i.e. lentil and methi, concurrent changes in Cd accumulation, growth, stress markers, components of antioxidant system and productivity of these legumes were studied.

Chapter-3

Materials and Methods



CONTENTS

MATERIALS AND METHODS

This chapter deals with the description of materials used for the study and methods adopted for the Experimentation and determination of various traits during the course of the investigation. The details of agro-climatic conditions, soil analysis, Experimentation, the techniques and procedure employed in this regard have been given below. In order to assess the synergistic effect of *Rhizobium* (R) and Arbuscular Mycorrhizal (AM) fungi to reduce the stress caused by cadmium (Cd) toxicity, pot Experiments were conducted on selected leguminous crops during the Rabi (winter) seasons of 2010-2013.

3.1 Botanical description and economic importance of crops

3.1.1 Methi (Trigonella foenum-graecum L.)

It is small, erect and 30 to 60 cm tall an annual herb. Leaves are pinnately compound, trifoliolate; leaflets are about 2.0 to 2.5 cm long, oblanceolate with dentate margin. Flowers are one to two, axillary in position and yellowish white in colour. The pods are 3 to15 cm long and with ten to twenty seeds. Seeds are greenish brown with hooked appearance due to the presence of deep groove across one corner. It is used as in the preparation of yellow dye, for cosmetics production, as green manure in agricultural and also as good soil renovator. Seeds and leaves are rich in minerals, protein and carbohydrate but low in oil content. Seeds are used as condiment and also in the treatment of chronic dysentery, diarrhoea, cough and enlargement of liver as well as spleen. They also contain the steroidal substance called diosgenin which is used as a starting material for the synthesis of oral contraceptives whereas leaves are used as vegetables.

3.1.2 Broad Bean (Vicia faba L.)

It is also faba bean and bakla in India. It is an annual herb with coarse upright stem and profusely branched taproot. The leaves are pinnately compound with 2 to 6 leaflets which do not have tendrils. Flowers are large, white or purple in colour and borne on short pedicle in a cluster of 1 to 7. They have five petals with standard petal white in colour and the wing petal is also white with a black spot and two white keels. Stamens are 10 in number. Pods are up to 40 cm long and 1to 3 cm in diameter. Each

pod contains three to ten seeds. Seeds are brown or black in colour compressed but gain on drying. Leaves are used as vegetable and as green fodder for hay.

3.1.3 Bengal gram (Cicer arietinum L.)

It is commonly known as Bengal gram and is the most important crop of this country. It is freely branched annual herb, 25 to 50 cm tall. Stems, leaves and fruits are covered with glandular hairs. Strong tap root and lateral roots with large lobed nodules are developed. Leaves are pinnately compound. Flower is solitary, axillary. Calyx is united with 5 toothed corollas, one cm long, greenish white to pink or blue in colour, stamens 9+1. Pods are inflated up to 3×2 cm with 1to 2 seeds. Seeds are angular up to one cm diameter. Germination of seed is hypogeal. It is mainly used for human consumption as well as feeding cattle and is also used as 'dal' in split for whole. Fried or boiled seeds are also eaten. Husk and bits of dal are used as nutritious feed for animals. Green leaves are also used as vegetable. It contains 18.2% protein, 62.0% carbohydrates, 4.0% fat and is a rich source of Ca, Fe and niacin.

3.1.4 Pea (Pisum sativum L.)

Pisum sativum is a cool season annual plant which is grown in many parts of the world. It is generally grown on loam and clay loams. It weights between 0.1 and 0.36 grams and has high nutritional value of its seed which are consumed both in the fresh form as vegetable and in the dried form as a pulse. In India, it is often split into dal and also used for making roasted or parched pea. They are rich source of starch, protein, vitamins particularly of the B group, and minerals. Sugar is present in the form of sucrose, stachyose, glucose, fructose and galactose.

3.1.5 Lentil (Lens culinaris Medik.)

Lentil is one of the most important leguminous crops grown in India and it is also known as masur. It is an erect light green, freely branched annual herb with 25 to 40 cm height freely branched with slender stem. It is an important leguminous pulse crop of winter season. It is grown in almost all parts of country as a pulse crop. It is an annual herb, erect and light green in colour. It has softly hairy foliage. It has a height of 25 to 40 cm. Pods are smooth and 1.3 cm long. It is used as dal both as whole grains and in splitted form.

3.2 Agro-climatic conditions of Aligarh

Aligarh has an area of about 5,024 sq. kms, situated in Western Uttar Pradesh at 27°52'N latitude, 78°51'E longitude, and 187.45 m altitude above sea level. Severe hot summers and intense cold winters prevail throughout the year. The winter extends from middle of October to end of March. The coldest month of winter is January whose mean temperatur13°C. The minimum recorded for any single day is 5°C. The summer extends from April to the end of June and the average temperature of June is about 34°C, whereas the extreme maximum can go upto 45.5°C. The monsoon extends from the end of June to middle of October. The mean annual rainfall is about 847.3 mm.

More than 85% of the total rainfall occurs during June to September and nearly 10% of it is useful for winter crops. The relative humidity in the winter ranges between 56% to 77% with an average of 66.5% and for summer the range is from 37% to 49% with an average of 43%; for the monsoon season, it ranges between 63% and 73% with an average of 68%.

3.3 Analysis of soil

To ensure maximum soil-aeration, thoroughly ploughed soil was collected from University Agriculture Farm, Aligarh Muslim University, Aligarh for the filling of earthen pots. Before sowing of seeds, soil samples were collected randomly from different pots for the analysis of selected soil-characteristics. The physio-chemical properties of the soil for each Experiment have been given in Table 1.

Table 3.1: Some physiochemical properties of the soil used in the present study.

Soil characteristics	Experiment 1 (2010-2011)	Experiment 2 & 3 (2011-2012)	Experiment 4 (2012-2013)
Texture	Sandy loam	Sandy loam	Sandy loam
pH	7.8	8.10	8.00
Available N (mg Kg^{-1} soil)	90.54	100.16	98.72
Available P (mg Kg^{-1} soil)	8.89	8.75	8.58
Available K (mg Kg^{-1} soil)	112.37	110.55	108.65
Total cadmium (mg Kg^{-1} soil)	0.310	0.300	0.323

3.4 Preparation of materials

3.4.1 Plant materials

The plant materials taken for screening to test the tolerance of Cd were methi (*Trigonella-foenum-graecum* L.; desi), broad bean (*Vicia faba* L.; VH-82-1), chick pea (*Cicer arietinum* L.; BG-472), pea (*Pisum sativum*; Kashi Udai) and lentil (*Lens culinaris*; Medik.; K-75). Methi and lentil were screened out for the subsequent Experiments (2, 3 and 4). Healthy and viable seeds of all the plants were procured from the National Research Centre on Plant Biotechnology (NRCPB) of the Indian Agricultural Research Institute (IARI), New Delhi, India.

3.4.2 Rhizobium and AM fungi

The certified and viable *Rhizobium* cultures specific for methi and lentil were procured from Indian Agricultural Research Institute (IARI) New Delhi. Seeds were surface sterilized with 0.01% mercuric chloride for 2 min and then washed three times with distilled water. The culture of *Rhizobium* was thoroughly mixed with sugar and water. Surface sterilized seeds were treated with this mixture and dried in shade before sowing (Subba Rao, 1972).

The culture of AM Fungi was procured from the Division of Microbiology, an extensive centre of Indian Agriculture Research Institute, New Delhi. The spores were applied at the rate of thousand spores per pot as a thin layer below the seeds before sowing.

3.4.3 Preparation of pots

Before the start of each Experiment, earthen pots (25cm diameter × 25cm height) were filled with soil thoroughly mixed with farmyard manure in 3:1 ratio. Four Kg soil was filled in each pot. Appropriate amount of 0, 163.2, 326.1, 489.2 and 652.2 mg $CdCl_2$ were mixed thoroughly with 4.0 Kg soil to achieve 0, 25, 50, 75, 100 mg Cd Kg^{-1} soil, respectively. In Experiment 1, all the plants were treated with different doses of Cd, whereas, in Experiment 2, sensitive and non-sensitive legumes were given the treatment of only moderate (50 mg Cd Kg^{-1}) and highest (100 mg Cd Kg^{-1}) dose of Cd alone or in combination with *Rhizobium*, while in Experiment 3, sensitive and non-sensitive legumes were giving the treatment of only moderate and highest dose of Cd alone or in combination with Cd or AM fungi, in Experiment 4; the two selected legumes were treated with 50 mg Cd Kg^{-1} and 100 mg Cd Kg^{-1}alone or in

combination with dual inoculation of *Rhizobium* + AM fungi. Plants grown without any treatment (no Cd, no AM fungi, and no *Rhizobium*) served as control.

3.4.4 Chemicals/reagents for analysis

For the analysis of various enzyme activities, chemicals were obtained from Sigma-Aldrich (St. Louis Mo, USA). Other major and minor salts and buffer components were procured from MERK, SRL. All chemicals used were of highest purity available.

3.5 Experimental set-up

Healthy seeds of 5 leguminous plants (methi, broad bean, chick pea, pea, and lentil,) were surface sterilized with 0.5% (v/v) sodium hypochlorite for 15 min and then rinsed three times with deionized water. Before seeds sowing, light application of tap water was given in each pot to provide necessary moisture for germination of the seeds. Ten seeds per pot were sown to avoid germination failure and after the establishment of seedlings, thinning was done to retain only four healthy plants of nearly equal size in each pot. Five sets of treatments (0, 25, 50, 75 or 100 mg Cd Kg^{-1}) each with four sets of plant (30, 60, 90 and 120 DAS) with three replicates were maintained. Each set of pots served as sampling material. Diagrammatic representation of the arrangement of pots for Experiment 1 has been shown in scheme 1. In Experiment 2, similar distribution of pots was made taking two screened plant into consideration. The distribution of pots for Experiment 2, 3 and 4 for determining various characteristics at 30, 60, 90 and 120 DAS has been described in appended schemes (2, 3 and 4).

3.6 Experimentation plan

The Experiments were carried out according to the standard agricultural practices. Calendar of various operations in each Experiment has been given in table 2, which shows time schedule for Experimentation, treatment and the data collection. Crop was irrigated with tap water as and when required. In order to check the pest, if any, insecticidal spray was done.

Table 3.2: Experimental calendar showing treatments and sampling for the three Experiments.

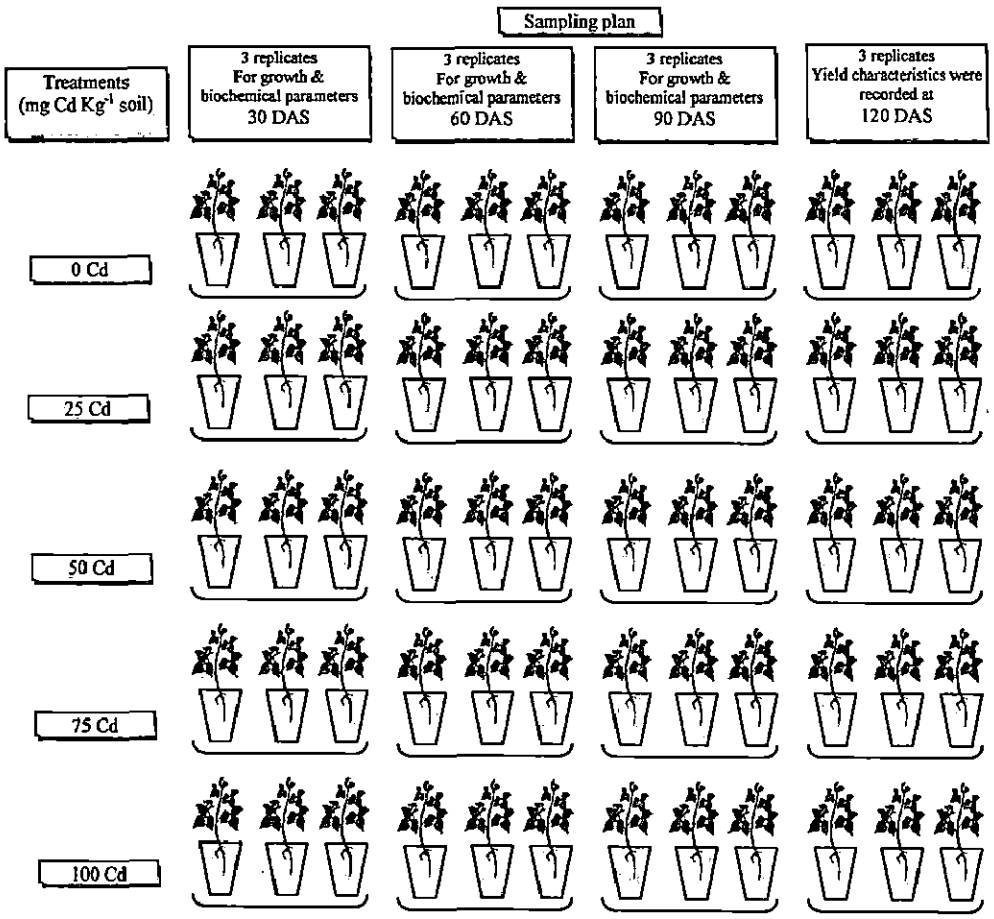
	Experiment 1 (2010-2011)	Experiment 2&3 (2011-2012)	Experiment 4 (2012-2013)
Preparation of pots	03.11.2010	06.11.2011	08.11.2012
Sowing	08.11.2010	10.11.2011	12.11.2012
Treatments			
	06.11.2010	08.10.203	08.10.2013
Cadmium	(0,25,50,75,100 mg Cd Kg^{-1}soil)	(0, 50, 100 mg Cd Kg^{-1}soil)	(0,50, mg Cd Kg^{-1}soil)
Replication	Three	Three	Three
Plants	methi, broad bean, chick pea, pea & lentil	Cd least-sensitive legume Cd most-sensitive legume	Cd least-sensitive legume Cd most-sensitive legume
Sampling stages			
Pre-flowering (30 DAS)	10.12.2010	09.12.2011	09.12.2012
Flowering (60 DAS)	10.1.2011	09.01.2012	09.01.2013
Fruiting (90 DAS)	10.2.2011	10.02.2012	10.02.2013
Harvest (120 DAS)	25.03.2011	23.03.2012	29.03.2013

3.7 Experiment 1

Experiment 1 was conducted the during winter season of 2010-2011. Surface sterilized seeds of five leguminous plants namely methi (*Trigonella-foenum-graecum*), broad bean (*Vicia faba*), chick pea (*Cicer arietinum*), pea (*Pisum sativum*) and lentil (*Lens culinaris*) were sown on November 8, 2010 and the crops were harvested at 120 DAS. The treatment in this Experiment was arranged in a factorial randomized block design. The aim of the Experiment was to study the effects of five levels of Cd doses (0, 25, 50, 75 and 100 mg Cd Kg^{-1}) on Cd accumulation, stress markers, growth, biochemical and yield characteristics to select Cd sensitive and Cd non-sensitive legumes. Samplings for all the attributes except yield was done at pre-flowering (30 DAS), flowering (60 DAS), post-flowering (90 DAS) stages while yield

Scheme 1

Diagrammatic representation of the arrangement of the pots for Experiment 1.



Scheme shows the arrangement of pots for one leguminous plant. Other leguminous plants also had similar arrangement of pots

characteristics were recorded at harvest (120 DAS). The scheme of treatments for Experiment 1 has been given in Table 2.

3.7.1 Parameters studied

Following parameters were studied at different sampling stages.

3.7.1.1 Growth characteristics

Plant height (cm)

Plant fresh mass (g)

Plant dry mass (g)

Leaf area per plant (cm^2)

Leaf number per plant

Number of nodules per root system

3.7.1.2 Physiological and biochemical characteristics

Total chlorophyll contents (mg $g^{-1}FW$)

Carbonic anhydrase (CA) activity (nm CO_2 $g^{-1}FW$ S^{-1})

Nitrate reductase (NR) activity (nm NO_2 $g^{-1}FW$ S^{-1})

Malondialdehyde (MDA) content ($nmolg^{-1}FW$)

Proline content (µmol $g^{-1}FM$)

Leaf protein content (mg $g^{-1}FM$)

Cadmium content in total plant (µg $g^{-1}DM$)

3.7.1.3 Yield characteristics

Pod length (cm)

Number of pods per plant

Number of seeds per pod

1000 seeds weight (g)

Seed yield per plant (g)

3.7.1.4 Toxicity index

3.8 Experiment 2

Experiment 2 was conducted on the basis of the findings of Experiment 1 during winter season of 2010-2011. The sowing of seeds of sensitive and non-sensitive plants were done on November 10, 2011 and were harvested on March 23, 2012. The treatments of this Experiment were arranged in factorial randomized block design. Seeds were coated with the broth of *Rhizobium* culture on the same day of sowing and they were sown in pots amended with 0, 50 and 100 mg Cd Kg^{-1} soil. The aim of the Experiment 2 was to assess the effect of *Rhizobium* application in mitigating the Cd-induced effects on growth, biochemical parameters, stress markers, components of antioxidant system, mineral acquisition in leaves, Cd accumulation in root and shoot and yield characteristics in Cd sensitive and Cd non-sensitive leguminous plants. The time of sampling was the same as for Experiment 1. The scheme of treatments for Experiment 2 has been given in Table 6.

3.8.1 Parameters studied

Following parameters were studied at different sampling stages.

3.8.1.1 Growth characteristics

Shoot and root lengths per plant (cm)

Fresh mass of shoot and root per plant (g)

Dry mass of shoot and root per plant (g)

Number of leaf per plant

Leaf area per plant (cm^2)

Number of nodules per root system

3.8.1.2 Physiological and biochemical characteristics

Chlorophyll a content (mg $g^{-1}FW$)

Chlorophyll b content (mg $g^{-1}FW$)

Total chlorophyll content (mg $g^{-1}FW$)

Carotenoid content (mg $g^{-1}FW$)

Carbonic anhydrase (CA) activity (nm CO_2 $g^{-1}FW$ S^{-1})

Nitrate reductase (NR) activity (nm NO_2 $g^{-1}FW$ S^{-1})

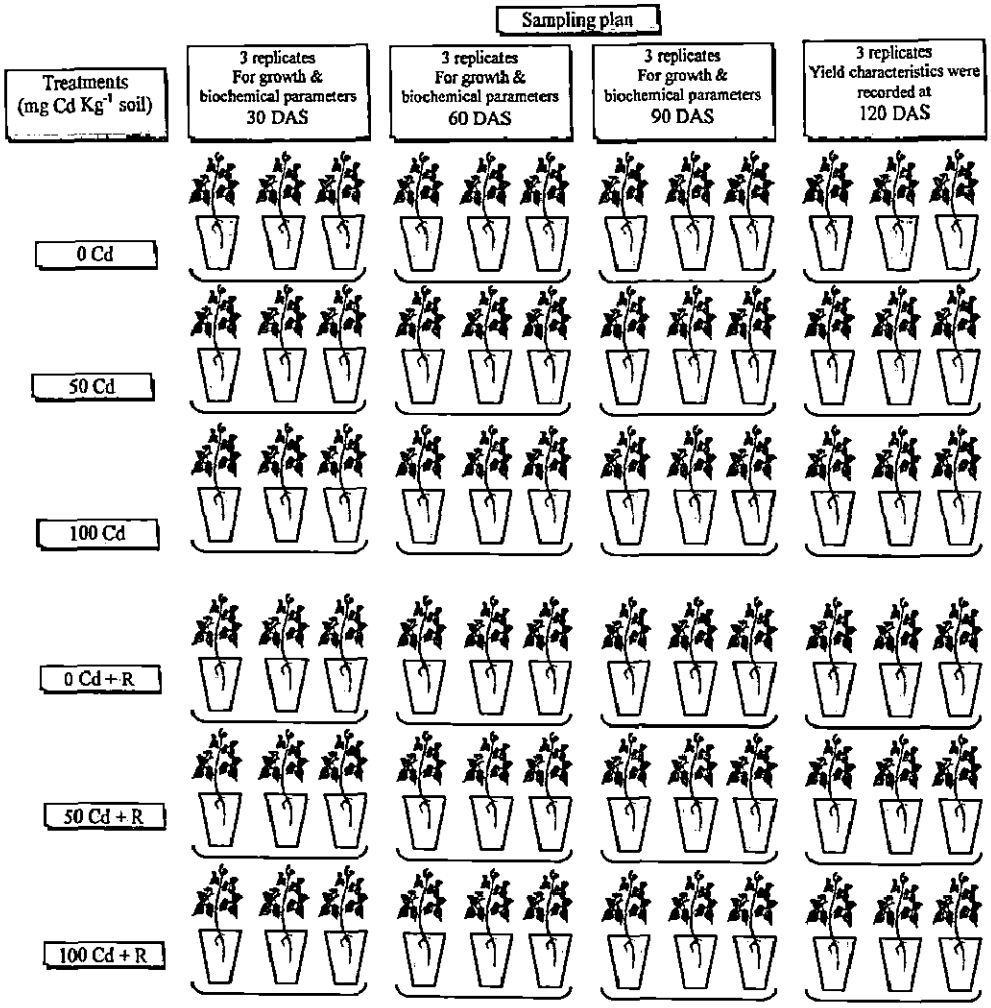
Leaf protein content (mg $g^{-1}FM$)

Malondialdehyde (MDA) content ($nmolg^{-1}FW$)

Proline content (µmol $g^{-1}FM$)

Scheme 2

Diagrammatic representation of the arrangement of the pots for Experiment 2.



Scheme shows the arrangement of pots for one leguminous plant. Other leguminous plant also had similar arrangement of pots

Peroxidase (POX) activity (U mg^{-1} protein)

Superoxide dismutase (SOD) activity (U mg^{-1} protein)

Catalase (CAT) activity (U mg^{-1} protein)

Plant (leaf) NPK content (%)

Root and shoot cadmium content (μg g^{-1} DW)

3.8.1.3 Yield characteristics

Pod length (cm)

Number of pods per plant

Number of seeds per pod

1000 seeds weight (g)

Seed yield per plant (g)

3.8.1.4 Per cent colonization of AM fungi (for Experiment 3 and 4)

3.8.1.5 Toxicity index

3.9 Experiment 3

Experiment 3 was conducted on the basis of the findings of Experiment 1 during winter season of 2010-2011. Seed sowing was done on November 10, 2011 and crop was harvested at 120 DAS. The treatments of this Experiment were arranged in factorial randomized block design. The aim of the Experiment 3 was to assess the effect of AM fungi application in the alleviation of Cd stress in Cd-sensitive and non-sensitive legumes. Culture of AM fungi was applied to the soil at the rate of 2gm per pot and treatments 0, 50 and 100 mg Cd Kg^{-1} soil were also given one day prior to sowing of seeds. The extent of alleviation of Cd stress by the treatment of fungi was assessed in terms of growth, biochemical characteristics, stress markers, component, of antioxidant system, Cd accumulation in root and shoot and yield characteristics. The time of sampling was the same as for Experiment 1. The scheme of treatments for Experiment 2 has been given in Table 6.

3.10 Experiment 4

Experiment 4 was conducted on the basis of the findings of Experiment 1 during winter season of 2012-2013. The sowing of seeds of sensitive and non-sensitive plant was done on November 15, 2012 and crop was harvested at 120 DAS. The treatments in this Experiment were arranged in a factorial randomized block design. The method of inoculation of *Rhizobium* was the same as that of the Experiment 2 while

application of AM fungi was the same as that of the Experiment 3 and the pots were treated with a same doses of Cd as in Experiment 2 and 3.The aim of the Experiment 4 was to assess the dual inoculation of *Rhizobium* and AM fungi application in the alleviation of Cd stress in Cd-sensitive and Cd non-sensitive leguminous plants. The extent of alleviation of Cd stress by the treatment of *Rhizobium* and AM fungi application assessed in terms of growth, biochemical characteristics, stress markers, components of antioxidant system, Cd accumulation in root and shoot and yield characteristics. The time of sampling was the same as for Experiment 1. The scheme of treatments for Experiment 2 has been given in Table 6.

Table 3.3: Scheme of the treatments for Experiment 1.

Leguminous plants	Treatments (mg Cd Kg^{-1} soil)				
	0 Cd	25Cd	50Cd	75Cd	100Cd
Methi	+	+	+	+	+
Broad bean	+	+	+	+	+
Chickpea	+	+	+	+	+
Pea	+	+	+	+	+
Lentil	+	+	+	+	+

Table 3.4: Scheme of the treatments for Experiment 2.

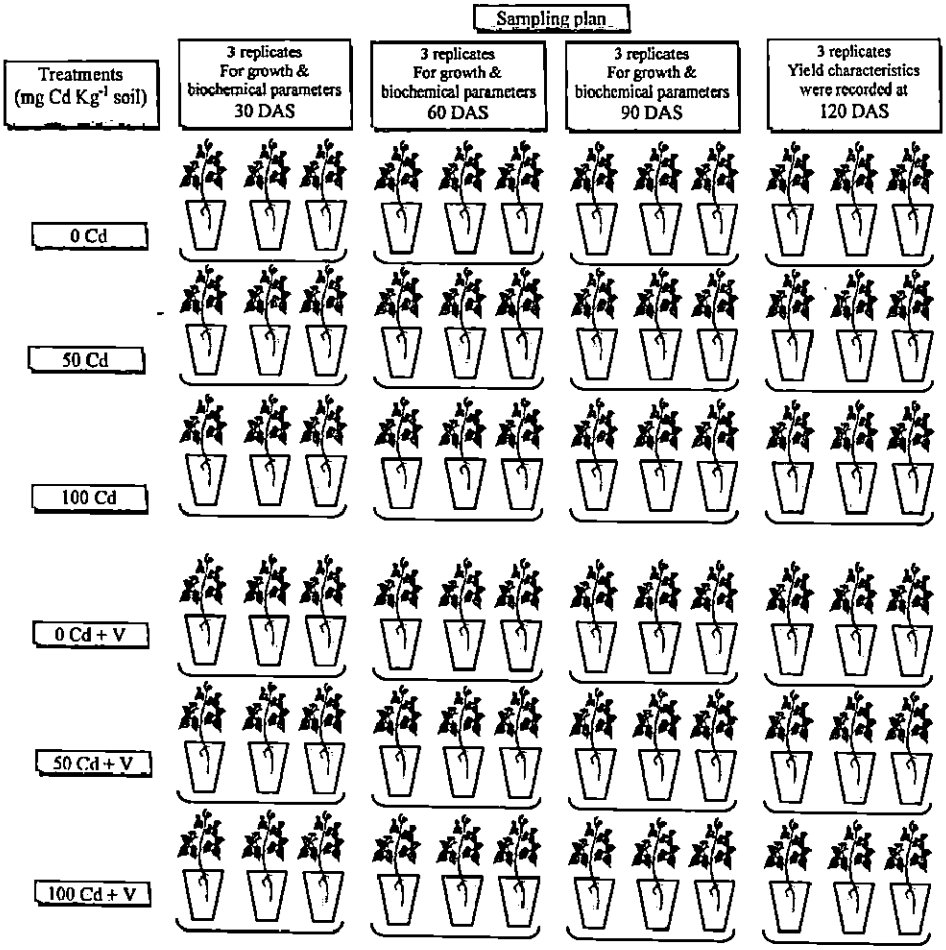
Treatments (mg Cd Kg^{-1} soil)	Leguminous plants	
	Methi (Cd non-sensitive)	Lentil (Cd sensitive)
0Cd	+	+
50Cd	+	+
100Cd	+	+
0Cd + *Rhizobium*	+	+
50Cd + *Rhizobium*	+	+
100Cd + *Rhizobium*	+	+

Table 3.5: Scheme of the treatments for Experiment 3.

Treatments (mg Cd Kg^{-1} soil)	Leguminous plants	
	Methi (Cd non-sensitive)	Lentil (Cd sensitive)
0Cd	+	+
50Cd	+	+
100Cd	+	+
0Cd + AM fungi	+	+
50Cd + AM fungi	+	+
100Cd + AM fungi	+	+

Scheme 3

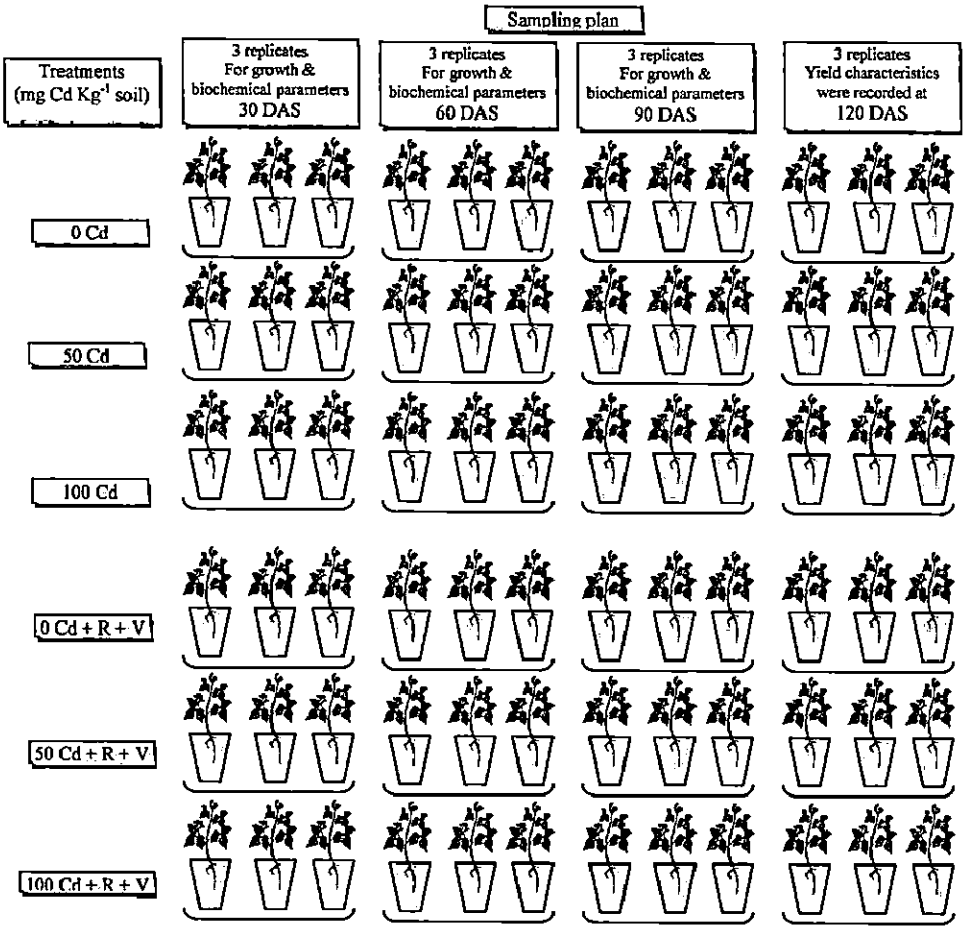
Diagrammatic representation of the arrangement of the pots for Experiment 3.



Scheme shows the arrangement of pots for one leguminous plant. Other leguminous plant also had similar arrangement of pots

Scheme 4

Diagrammatic representation of the arrangement of the pots for Experiment 4.



Scheme shows the arrangement of pots for one leguminous plant. Other leguminous plant also had similar arrangement of pots

Table 3.6: Scheme of the treatments for Experiment 4.

Treatments (mg Cd Kg^{-1} soil)	Leguminous plants	
	Methi (Cd non-sensitive)	Lentil (Cd sensitive)
0Cd	+	+
50Cd	+	+
100Cd	+	+
0Cd + AM fungi+*Rhizobium*	+	+
50Cd+AM fungi+*Rhizobium*	+	+
100Cd+AM fungi+*Rhizobium*	+	+

3.11 Methodology

3.11.1 Growth characteristics

Plants were uprooted and length of the main tap root and shoot lengths measured on a meter scale. To assess weight mass, three plants from each treatment, already evaluated for various parameters were dried for about 72 hours in hot air oven maintained at 80°C and then weighed. The weights of the sample were recorded with the help of electronic balance (CY204, Scalteo Ins., Germany). After separating all the leaves at petiole and stem junction, leaf area per plant was recorded with help of a leaf area meter (LA211, Systronics, Ahmedabad, India).

3.11.2 Physiological and biochemical characteristics

3.11.2.1 Chlorophyll a, b, total chlorophyll and carotenoids content

Chlorophyll a, chlorophyll b, total chlorophyll and carotenoid contents in fresh leaves were estimated by the method of Lichtenthaler and Buschmann (2001). Hundred mg of fresh leaves were taken from interveinal area and ground in 10 ml of 80% acetone (Appendix 1.1), by using a mortar and pestle. The suspension was decanted and filtered through a Whatman filter paper No.1 into a Buchner funnel. The optical density (OD) of the solution was read at 645 and 663 nm for chlorophyll estimation and at 480 and 510 nm for carotenoid estimation by spectrophotometer (UV-1700, Shimadzu, Japan). The chlorophyll a, chlorophyll b total chlorophyll and carotenoid contents were calculated using the following formula.

Chlorophyll a content = 12.7 (OD 663) + 2.69 (OD 645) × $\frac{V}{W\times1000}$ mg g^{-1} FW

Chlorophyll b content = 22.9 (OD 645) + 4.68 (OD 663) × $\frac{V}{W\times1000}$ mg g^{-1} FW

Total chlorophyll content = 20.2 (OD 645) + 8.02 (OD 663) × $\frac{V}{W\times1000}$ mg g^{-1} FW

Carotenoid content = 7.6 (OD 480) – 1.49 (OD 510) × $\frac{V}{d\times W\times1000}$ mg g^{-1} FW

Where,

OD = Optical density of the extract at given wavelengths (645, 663, 480 and 510 nm)

V = Final volume of chlorophyll extract in 80 % acetone

W = Fresh weight of leaf tissue (g)

d = Length of light path = 1 cm

3.11.2.4 Malondialdehyde (MDA) level

The level of lipid peroxidation product in the leaves was determined as Malondialdehyde content by a modified version of the method described by Cakmak and Horst (1991).

Estimation

0.5 gm of fresh leaf was weighed and ground in 10 ml of 0.1% (w/v) trichloroacetic acid (TCA) (Appendix 2.2) and centrifuged at 15,000 rpm for 5 minutes. 1 ml of the supernatant was taken in a separate test tube and 4.0 ml of 0.5% 2-Thiobarbituric acid (TBA) made in 20% (w/v) trichloroacetic acid was added (Appendix 2.3). The mixture was heated at 95°C for 30 minutes, cooled quickly in an ice bath and centrifuged at 12,000 rpm for 5 minutes. The absorbance of the supernatant was read at 532 nm on a spectrophotometer and corrected for non-specific turbidity by subtracting the absorbance at 600 nm. The value obtained was used for calculation by the following formula:

MDA concentration = $\frac{(A_{532}-A_{600})\times V\times1000}{\epsilon\times W}$

Where,

ϵ=specific extinction coefficient (155 mM^{-1} cm^{-1})

V=volume of extraction medium

W=fresh weight of leaf

A=absorbance at specific wavelength

The activity of the enzyme was expressed in terms of nmol g^{-1} fresh weight.

3.11.2.3 Leaf proline content

The proline content in fresh leaves was estimated by the method used by Bates et al. (1973). Fresh sample (0.5 g) was homogenized in a mortar with 5 cm^3 of 3% sulphosalicylic acid (Appendix 3.1). The homogenate was filtered through Whatman filter paper No. 2 and collected in a test tube with two washings. Five cm^3 of sulphosalicyclic acid, 2 cm^3 each of glacial acetic acid and acid ninhydrin (Appendix 3.2) were added to 2 cm^3 of the above extract. This mixture was heated in boiling water bath for 1 hour. The reaction was terminated by transferring the test tubes to ice box. Four cm^3 of toluene was mixed in the reaction mixture with vigorous shaking for 20-30 s. The chromophore (toluene) layer was aspirated and warmed at room temperature. The absorbance of red colour was read at 520 nm against a reagent blank. The amount of proline in the sample was calculated by using a standard curve prepared from pure proline (range, 0.1-36 µmol) and expressed on fresh mass basis of the sample.

$$\mu \text{ moles of proline } g^{-1} \text{ tissue} = \frac{\mu g \text{ proline } cm^{-3} \times cm^{-3} \text{ toluene}}{115.5} \times \frac{5}{g \text{ (sample)}}$$

Where, 115.5 is the molecular mass of proline

3.11.2.4 Antioxidant enzymes activity

Leaf tissue (500 mg) was homogenized in 5 cm^3 of 50 mM phosphate buffer (pH 7.0) (Appendix 4.1), containing 1% polyvinyl pyrrolidone. The homogenate was centrifuged at 15,000 rpm for 10 minutes at 5°C and the supernatant was used for the estimation of peroxidase, catalase and superoxide dismutase activities.

3.11.2.4.1 Leaf peroxidase (POX) activity

The POX activity was measured following the method of Chance and Maehley (1956) in fresh leaf samples. Three cm^3 of pyrogallol phosphate buffer (Appendix 5.1), 0.1 cm^3 of enzyme extract and 0.5 cm^3 of 1% H_2O_2 were mixed in a cuvette. Change in the absorbance, at 20 s interval for a period of 3 minutes was recorded at 420 nm on a spectrophotometer. The control set was prepared by boiling the enzyme extract.

3.11.2.4.2 Leaf catalase (CAT) activity

The activity of CAT was estimated by permanganate titration method (Chance and Maehly, 1956). Five cm^3 of phosphate buffer (Appendix 6.1), 1 cm^3 of 0.1M H_2O_2 (Appendix 6.2) and 1 cm^3 of enzyme extract were mixed and incubated at 25°C for 1 minute. Then 10 cm^3 of 2% H_2SO_4 (Appendix 6.3) was added in it. The mixture was titrated against 0.1N potassium permanganate (Appendix 6.4) to find the residual H_2O_2 until a purple colour persists for at least 15 s. Similarly, a control set was maintained in which the enzyme activity was stopped by the addition of H_2SO_4 prior to the addition of the enzyme extract.

3.11.2.4.3 Leaf superoxide dismutase (SOD) activity

The activity of SOD was measured by the method of Beauchamp and Fridovich (1971). To the reaction mixture 1 cm^3 of 50 mM phosphate buffer (Appendix 7.1), 0.5 cm^3 of 13 mM methionine (Appendix 7.2), 0.5 cm^3 of 75 mM NBT (Appendix 7.3), 0.5 cm^3 of 0.1 mM EDTA (Appendix 7.5) and 0.1 cm^3 of the enzyme extract and at last 0.5 cm^3 of 2 µM riboflavin (Appendix 7.4) was added. The absorbance of the reaction mixture was read at 560 nm on a spectrophotometer.

3.11.2.5 Carbonic anhydrase (CA) activity

The enzyme carbonic anhydrase catalyses the reversible hydration of carbon dioxide (CO_2) to give the bicarbonate ion:

$$H_2O + CO_2 \xrightleftharpoons{CA} H^+ + HCO_3^-$$

The activity of the enzyme was determined by the method of Dwivedi and Randhawa (1974). The leaves were cut into small pieces of nearly one square cm at temperature below 25°C. Two hundred mg of them were taken and kept in a petridish containing 10 ml of 0.2M aqueous cystein solution (Appendix 8.1) at a temperature from 0 to 4°C for 20 minutes and again cut in further smaller pieces. The solution adhering on the leaf pieces was removed with the help of a blotting paper and transferred immediately into a test tube containing 4 ml of phosphate buffer of pH 6.8 (Appendix 8.2). In this test tube, 4 ml of 0.2 M sodium bicarbonate ($NaHCO_3$) in 0.02 M sodium hydroxide (NaOH) solution (Appendix 8.3) and 0.2 ml of 0.002% bromothymol blue indicator (Appendix 8.4) were added. After shaking, the tube was kept at 0 to 4°C for 20 minutes. Carbon dioxide liberated during catalytic action of

enzyme on $NaHCO_3$ was estimated by titrating the reaction mixture with 0.05N hydrochloric acid (Appendix 8.5), by using methyl red (Appendix 8.6) as an indicator. A control reaction mixture (without leaf pieces) was also titrated against 0.05N hydrochloric acid. The difference of sample reading and control reading was noted for further calculation of enzyme activity.

$$\frac{V \times 22 \times N\ \mu M\ CO_2\ mg^{-1}\ 20\ min^{-1}}{W}$$

Where,

V = difference in volume (ml) of hydrocholoric acid used in the control and sample

22 = equivalent weight of CO_2

N = normality of HCl (36.5)

W = weight (mg) of leaf

The activity of the enzyme was expressed in terms of mM CO_2 Kg^{-1} (leaf FM) s^{-1}.

3.11.2.6 Nitrate reductase (NR) activity

The activity of nitrate reductase was estimated by the intact tissue method of Jaworski (1971) based on the reduction of nitrate to nitrite as per the following biochemical reaction:

$$NO_3^- + NADH + H^+ \xrightarrow{NR} NO_2^- + NAD^+ + H_2O$$

The nitrite formed in the reaction was determined with the help of spectrophotometer. The leaves were cut into small pieces one square cm size, weighed and then transferred into plastic vials. To each vial, 2.5 ml of phosphate buffer of pH 7.5 (Appendix 9.1) and 0.5 ml of 0.2 M potassium nitrate solution (Appendix 9.2) were added followed by the addition of 2.5 ml of 5% isopropanol (Appendix 9.3). Two drops of chloramphenicol solution was added to avoid bacterial growth in the medium and incubated in a BOD incubator for 2h at 30±2°C in dark.

Development of colour

0.4 ml of incubated mixture was taken into a test tube, into which 0.3 ml of 1% sulphanilamide solution (Appendix 9.4) and 0.02% NED-HCl (Appendix 9.5) was added. The test tube was left for 20 minutes at room temperature for maximum colour development. The mixture was diluted to 5 ml with double distilled water (DDW).

The OD was read at 540 nm on the spectrophotometer. A blank was run simultaneously with each sample.

Standard curve for NRA

30 mg sodium nitrite ($NaNO_2$) was dissolved in 100 ml DDW. Of this solution, 0.8 ml was taken into a 100 ml volumetric flask. The volume was made up to 100 ml using DDW. From this diluted solution, ten aliquots, measuring 0.2, 0.4, 0.6, 0.8, 1.0, 1.2, 1.4. 1.6, 1.8 and 2.0 ml were taken into separate test tubes. Into each test tube, 0.3 ml each of 1% sulphanilamide and 0.02% NED-HCl was added in each test tube. The solution was diluted to 5 ml with DDW and OD was read at 540 nm with the help of spectrophotometer. A blank was also run simultaneously. A standard curve was plotted between different concentrations of pure $NaNO_2$ versus OD of the solution. The OD of the sample was compared with a calibrated curve and NRA was expressed as nm NO_2 Kg^{-1} FW s^{-1}

3.11.2.7 Estimation of leaf protein content

The total protein content in leaves was estimated by the method of Lowry et al., (1951) 50 mg of the oven dried leaf powder was taken in a centrifuge tube and 5 ml of 5% trichloroacetic acid was added to it (Appendix 10.1) and allowed to stand for 30 minutes at room temperature with thorough shaking for complete precipitation of the protein and centrifuged at 4,000 rpm for 15 minutes, the supernatant was discarded. 5 ml of 1N sodium hydroxide (Appendix 10.2) was added to the residue and mixed well. It was left for 30 minutes on water bath at 60°C so that all the precipitated protein may completely get dissolved. After cooling for 15 minutes, the mixture was centrifuged at 4,000 rpm for 15 minutes and the supernatant containing protein fraction together with three washing with 1N NaOH was collected in 25 ml volumetric flask. Volume was made up to the mark with 1N NaOH and used for the estimation of protein.

1ml sodium hydroxide extract was transferred to 10 ml test tube and 5 ml reagent-C (Appendix 10.3) was added. The solution was mixed well and allowed to stand for 15 minutes at room temperature. 0.5ml Folin's phenol reagent (Appendix 10.4) was added rapidly with immediate mixing. The blue colour developed was left for 30 minutes for maximum colour development. Absorbance of this solution was read at 660 nm.

A blank containing DDW, reagent-B and Folin's phenol reagent was run simultaneously with each sample. The protein content were calculated by comparing the optical density of each sample with calibration curve plotted by taking known graded dilutions of standard solution of Bovine serum albumin (Fraction-V) and the leaf protein contents was expressed in terms of percentage on dry weight basis.

Standard curve for total proteins

50mg bovine serum albumin (Fraction-V) was dissolved in 50 ml DDW 10 ml of this solution was diluted to 50 ml. 1 ml of this solution contains 200 µg proteins. From this 0.2, 0.4, 0.6, 0.8 and 1.0 ml solution was transferred to 15 test tubes separately. The solution in each test tube was diluted to 1 ml with DDW. A blank of 1 ml DDW was also run with each set of determination. 5 ml reagent-B was added to each test tube including blank, was mixed well and allowed to stand for 10 minutes. To this solution 0.5 ml Folin phenol reagent was added and mixed well and incubated at room temperature in the dark for 30 minutes. Blue colour developed was read at 660nm.

3.11.2.8 Leaf N, P and K contents

The leaf samples were dried in hot-air oven at 80°C for forty-eight hours. Dried leaves were finely powdered and the powder thus obtained was sieved. The powder was labelled and stored in small polythene bags for the analysis.

Digestion of leaf powder

Sample powder was digested according to Lindner (1944) for the estimation of N, P and K. Hundred mg oven-dried leaf powder was transferred into a 50 ml Kjeldahl flask to which 2 ml of concentrated sulphuric acid was added. The flask was heated on a temperature controlled assembly for about two hours to allow complete reduction of nitrate present in the plant material by organic matter itself. As a result, the content of the flask turned black. After cooling the flask for about 15 minutes at room temperature, 0.5 mL of 30% hydrogen peroxide (H_2O_2) was added drop by drop and the solution was heated again till the colour of the solution changed from black to light yellow, 3-4 drops of 30% H_2O_2 were added after cooling for about 30 minutes. followed by heating for another 5 minutes. The addition of 30% H_2O_2 followed by heating was repeated until the content of the flask turned colourless. The peroxide-digested material was transferred from the Kjeldahl flask to a 100 ml volumetric flask with three washings each with 5ml of DDW. The volume of flask was then made up

to the mark with DDW. This aliquot was used to estimate N, P and K contents. The details of methods used for the analysis of these elements are given below separately.

3.11.2.8.1 Nitrogen

Nitrogen content was estimated according to the method of Lindner (1944). Ten ml aliquot of the digested material was taken into a 50 ml volumetric flask. To this, 2 ml of 2.5 N sodium hydroxide (Appendix 11.1) and 1 ml of 10% sodium silicate solution (Appendix 11.2) were added to neutralize the excess of acid and to prevent turbidity respectively. The volume was made up to the mark with DDW, 5 ml aliquot of this solution was taken into a 10 ml graduated test tube and 0.5 ml Nessler's reagent were added. The test tubes were allowed to stand for 5 minutes for maximum colour development. The solution was transferred into a spectrophotometric tube and the OD of the solution was read 525 nm by using spectrophotometer at. A blank was also run simultaneously. The reading of each sample was compared with the standard calibration curve and nitrogen was expressed in the terms of percentage on dry weight basis.

Standard curve

50 mg pure ammonium sulphate was dissolved in a sufficient volume of DDW and the final volume was made up to 1 litre with DDW. From this solution, 0.1, 0.2, 0.3, 0.4, 0.5, 0.6, 0.7, 0.8, 0.9 and 1.0 ml solution were pipetted into ten test tubes separately. The solution in each test tube was diluted to 5 ml with DDW. In each test tube, 0.5 ml Nessler's regent was added. After 5 minutes, the OD was read at 525 nm by using the spectrophotometer. A blank was also run side by side. Standard curve was plotted by using known graded concentrations of ammonium sulphate solution versus OD.

3.11.2.8.2 Phosphorus

The method of Fiske and Subbarow (1925) was used to estimate the total phosphorus in digested material. 5 ml aliquot was taken in a 10 ml graduated test tube and 1 ml of 2.5% molybdic acid (Appendix 11.3) was added carefully followed by addition of 0.4 ml 1-amino-2-naphthol-4 sulphonic acid (Appendix 11.4). When the colour turned blue, the volume was made up to 10 ml with DDW. The solution was shaken for 5 minutes and the OD was read on the spectrophotometer at 620 nm. A blank was also run side by side.

Standard curve

351 mg pure potassium dihydrogen orthophosphate was dissolved in sufficient DDW, 10 ml of 10 N sulphuric acid was added to it and the final volume was made up to one litre with DDW. From this solution, 0.1, 0.2, 0.3, 0.4, 0.5, 0.6, 0.7, 0.8, 0.9 and 1.0 ml aliquots were taken into ten test tubes separately. The solution in each test tube was diluted to 10 ml with DDW. Into each test tube, 1 ml molybdic acid and 0.4 ml 1-amino- 2-naphthol-4- sulphonic acid were added. After 5 minutes, the OD of the solution was read at 620 nm. A blank was also run simultaneously. The standard curve was plotted by using different dilutions of potassium dihydrogen orthophpsphate versus OD. The percent phosphorus content was determined on dry weight basis.

3.11.2.8.3 Potassium

Potassium content was analyzed with the help of flame-photometer (Hald, 1946). In it, the peroxide-digested aliquot is discharged through an atomizer in the form of a fine mist into a chamber, where it is drawn in to a flame. Combustion of K produces light of 767 nm wavelength (i.e. in the violet range). The light produced was conducted through the appropriate filters to impinge upon a photoelectric cell that activates a galvanometer. The air was supplied through an air pump and liquid petroleum gas was used for combustion. The chimney of the equipment was removed and the gas was ignited by electric lighter. The final pressure of the two gases was adjusted to 15 pounds per inch. When the flame formed sharp blue cones, the correct filter was set and DDW was introduced by a beaker, the galvanometer was set to zero and standard solution of the element was sucked through a capillary tube. Now the galvanometer was adjusted to the 100 position by using the amplifier. Unless the 0 and 100 points are maintained on successive readings, the gas pressure, air-pressure or both were adjusted to bring about a stable position. Therefore, intermediate standards i.e. diluted solutions of known concentrations between 0 and 100 percent were checked and a graph was prepared. The relationship between the galvanometer readings and the concentrations appear in a curvilinear fashion rather than in straight line. At last, the samples were run and exact concentration of the element was calculated with the help of graph.

3.11.3 Cadmium accumulation estimation

The tissue samples the root and shoot were immersed for 10 min in ice cold 5 mM $CaCl_2$ solution for the estimation of Cd content (Appendix 12). In order to displace extracellular Cd, the samples were rinsed with distilled water and were oven dried (Meuwly and Rauser, 1992). Cd and samples were digested in nitric acid:perchloric acid (3:1, v/v). Cd content was determined by atomic absorption spectrophotometer (Perkin-Elmer A, Analyst, 300).

3.11.4 Yield Characteristics

Yield is the final manifestation of morphological, physiological and biochemical traits of a crop. At harvest (120 DAS), yield parameters were recorded. At harvest, pods were collected for measuring their length and number per plant. The number of seeds per pod was counted and their mean was taken. From the produce of the pots, a sample of thousand seeds were randomly drawn and weighted to compute seed yield. The total seeds from a plant in each treatment were cleared, sun-dried and weighed to compute seed yield per plant.

3.11.5 Percent AM fungi colonization

Plants roots were washed, nodules were counted and the roots were preserved in formalin-aceto-alcohol to determine the % mycorrhizal infection by the slide technique (Nicolson 1960) at 60 days after sowing of seeds. The root samples were cut into small segments, (1 cm) cleared in 10% KOH and stained with trypan blue lactophenol (Philips and Hayman, 1970). Roots segments were checked for AMF infection and the percent root colonization was calculated as follows:

$$Root\ colonization\ (\%) = \frac{\text{Number of AM positive segments}}{Total\ number\ of\ segments} \times 100$$

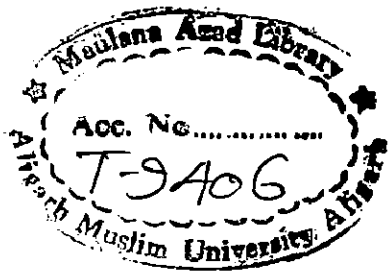
3.11.6 Tolerance index

Tolerance index of legumes was calculated by the following of formula Khan et al. (2006).

$$Tolerance\ Index = \frac{Seed\ yeild\ of\ treated\ plants}{Seed\ yield\ of\ control\ plants} \times 100$$

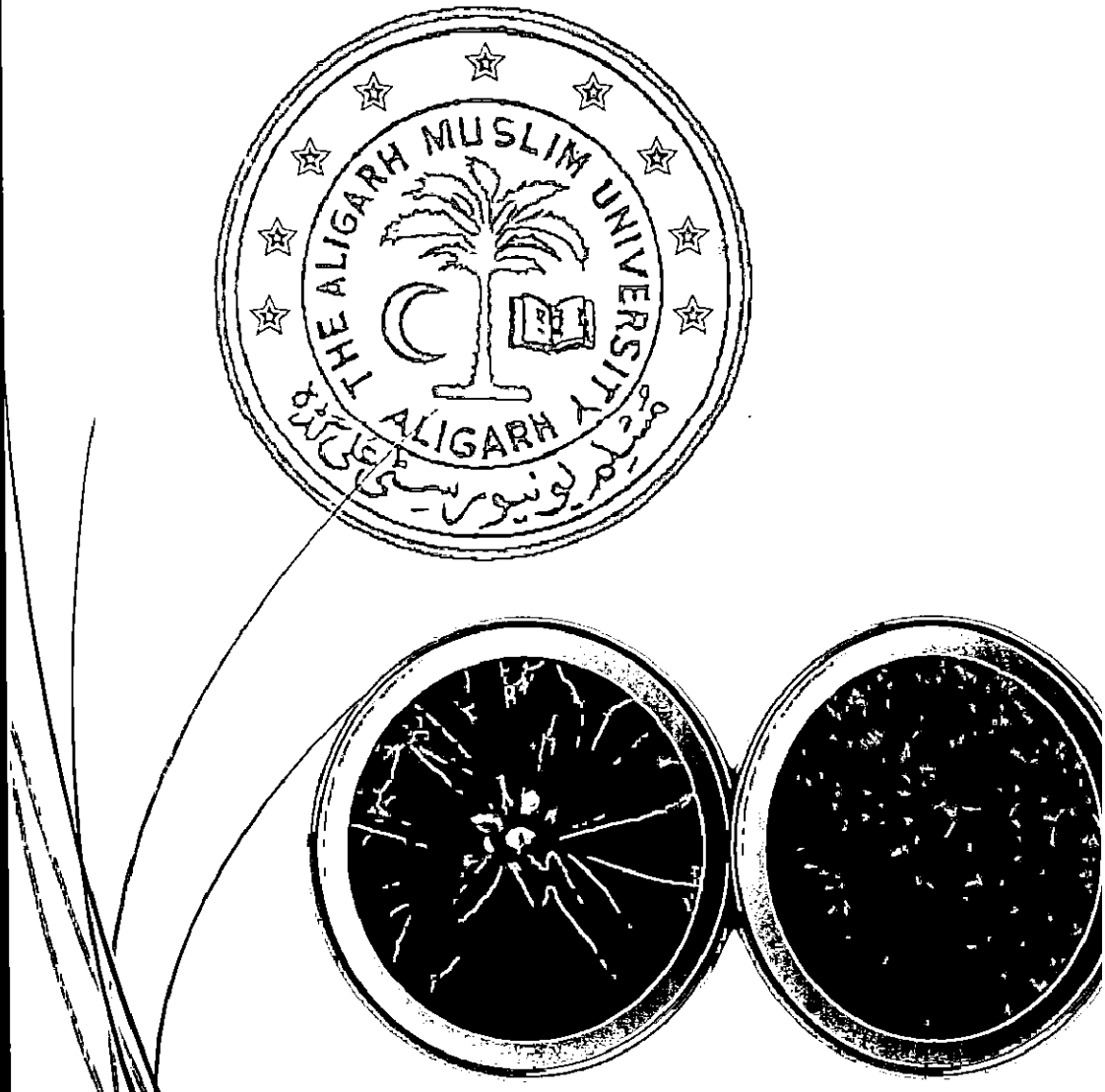
3.12 Statistical Analysis

Data were analyzed statistically using the Statistical Package for the Soil Sciences (SPSS, 10.0 for Windows). Standard error was calculated and analysis of variance was performed on the data to determine least significant difference (LSD) for significant data to identify difference in the mean of the treatment. The treatment means were separated using LSD test. Different letters indicate significant difference at $P < 0.05$.



Chapter-4

Experimental Results



CONTENTS

EXPERIMENTAL RESULTS

Experimental results presented herein mainly as changes in growth, biochemical characteristics, Cadmium (Cd) accumulation, stress markers, components of antioxidant system, per cent colonization of Arbuscular mycorrhizal (AM) fungi and yield attributes of leguminous plants as influenced by different doses of Cd, inoculation of *Rhizobium* and application AM fungi as well as dual inoculation of both the symbionts, per cent colonization and tolerance index was also calculated.

4.1 Experiment 1: Screening of legumes against different doses of soil amended with Cd

This Experiment was carried out to study the effects of five doses of Cd viz. 0, 25, 50, 75 or 100 mg Cd Kg^{-1} soil on growth, biochemical characteristics, stress markers and Cd accumulation in plant five leguminous plants, namely methi, broad bean, chick pea, pea and lentil at three growth stages i.e. 30, 60 and 90 DAS and yield attributes were recorded at the time of harvest. Tolerance index of all the plants was also calculated and legumes were designated as 'Cd-least sensitive' or 'Cd-most-sensitive' on the basis of their performance under Cd stresses (Figure 4.13).

The details of results are briefly described below and summarized in Figures (4.1 – 4.19).

4.1.1 Growth characteristics

The increasing doses of Cd decreased the growth characteristics (length, fresh and dry weights of plant and number of nodules per root system) of all the five legumes at all the three sampling stages. A significant reduction in growth parameters (plant length, plant fresh and dry weights and number of nodules per root system) was recorded with in different legumes at all the stages (30, 60 and 90 DAS) of growth (Figures 4.1- 4.6). The pattern of growth reduction in the five legumes was in order of lentil>pea>chick pea>broad bean >methi. Application of 100 mg Cd Kg^{-1} soil caused maximum reduction in growth characteristics followed by 75, 50 and 25 mg Cd Kg^{-1} soil for all the growth stages. Among legumes, methi showed less decrease in growth than lentil followed by broad bean, chick pea, pea and lentil. Reduction was maximum at an early growth stage i.e. 30 DAS than later stage i.e. 90 DAS (Figures 4.4 - 4.5). Plant length was decreased by 42.0, 61.8% at 30 DAS; 33.5, 59.8% at 60

.S and 20.1, 28.2% at 90 DAS in methi and lentil respectively, over their respective trols. A reduction of 53.7, 57.5% in fresh weights and 69.3, 73.3%, in dry weights to 100 mg Cd Kg^{-1} soil, over the control at 30 DAS was observed in methi and il respectively,. Lentil showed a decrease of 40.2, 60.0, 69.7 and 77.7% in number odules per root system due to increasing doses of Cd viz. 25, 50, 75 or 100 mg Cd $^{-1}$ soil respectively, over the control at 30 DAS.

.2 *Total chlorophyll content*

al chlorophyll content of the five leguminous plants decreased significantly with increasing doses of Cd at all the growth stages (Figure 4.7). Reduction in orophyll content in leaves increased with the increasing doses of Cd in all the uminous plants. The per cent reduction in chlorophyll content increased with age plants and was in order: lentil >pea >chick pea >broad bean >methi (Figure 4.8). interaction of Cd treatments with legumes was significant as compared to non-ted control plants. A reduction of 64.4% and 42.1% in chlorophyll content was erved in lentil and methi respectively, over the control at 30 DAS due to100 mg Kg^{-1} soil.

.3 *Lipid peroxidation and proline content*

id peroxidation and proline content were estimated to assess the extent of stress in erent legumes against Cd treatments viz. 25, 50, 75 or 100 mg Cd Kg^{-1} soil at erent stages of growth i.e. 30, 60 and 90 DAS of plant growth (Figures 4.12 - 3). Lipid peroxidation was calculated in terms of malondialdehyde (MDA) content he leaves of legumes. Per cent lipid peroxidation increased with the increase in the el of Cd content in soil being maximum at 100 mg Cd Kg^{-1} soil. MDA content wed an increase of 12.7, 24.9 ; 21.4, 29.9; 25.9, 37.2 and 33.4, 44.9% in methi and il respectively, over the control due to 25, 50, 75 and 100 mg Cd Kg^{-1} soil at 30 .S. So this parameter shows maximum values in methi followed by broad bean, ck pea, pea and lentil.

Proline content significantly enhanced with the increase of Cd stress ($CdCl_2$; 25, 50, 75 or 100 mg Kg^{-1} soil) and it corresponds to the pattern of lipid oxidation (Figure 4.13). Proline content showed an increase of 33.7, 41.3% at 30 .S and 26.8, 38.9% at 90 DAS was recorded in methi and lentil respectively, over ir respective control.

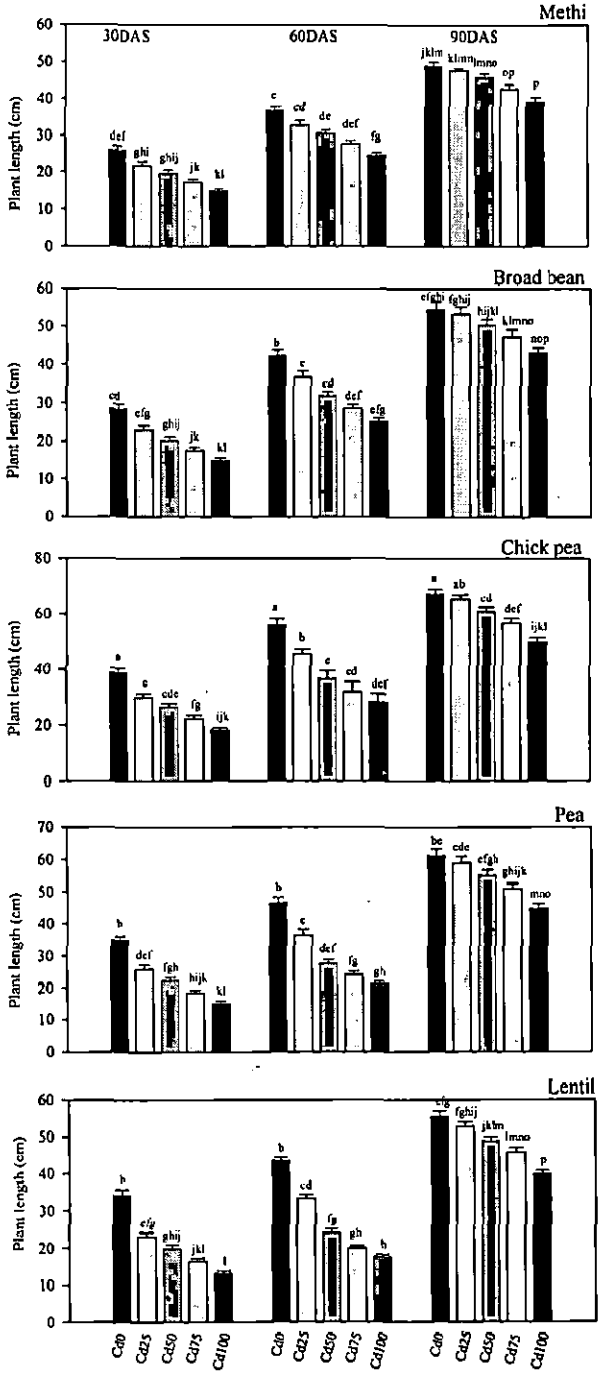


Figure 4.1: Effect of cadmium chloride ($CdCl_2$: 0, 25, 50, 75 and 100 mg Kg^{-1}) on the plant height (cm) of five legume crops at 30, 60 and 90 days after sowing.

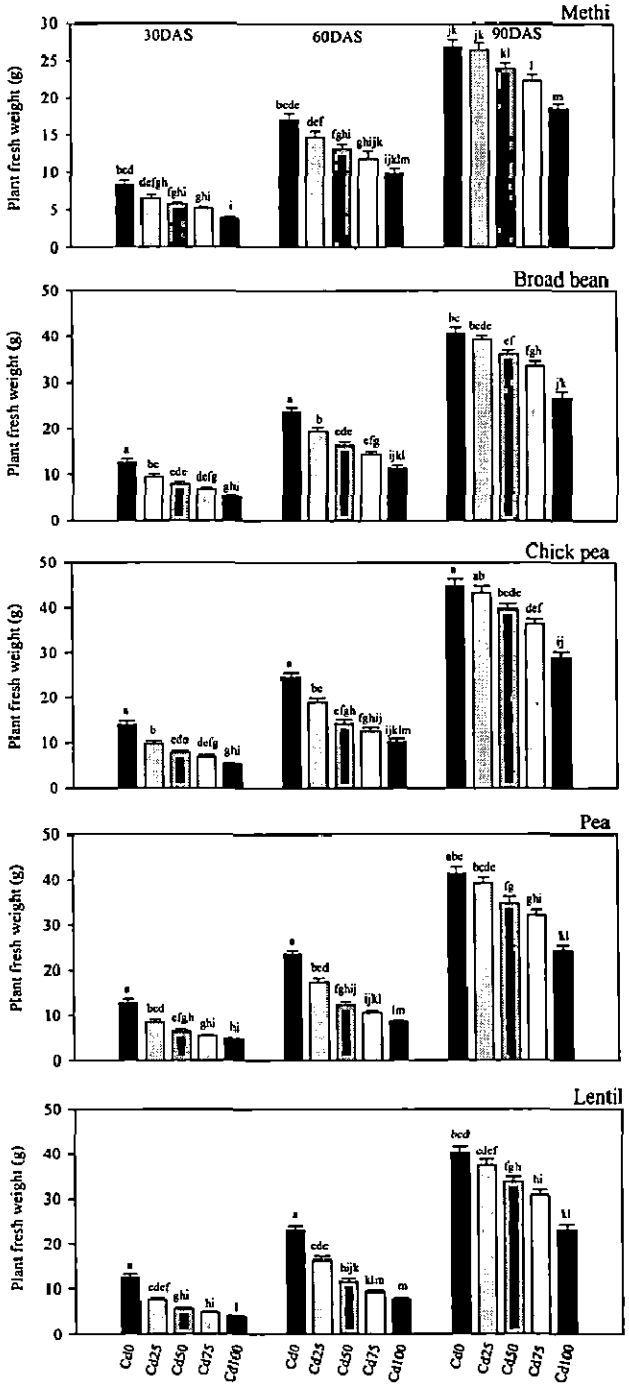


Figure 4.2: Effect of cadmium chloride ($CdCl_2$: 0, 25, 50, 75 and 100 mg Kg^{-1}) on the plant fresh weight (g) of five legume crops at 30, 60 and 90 days after sowing.

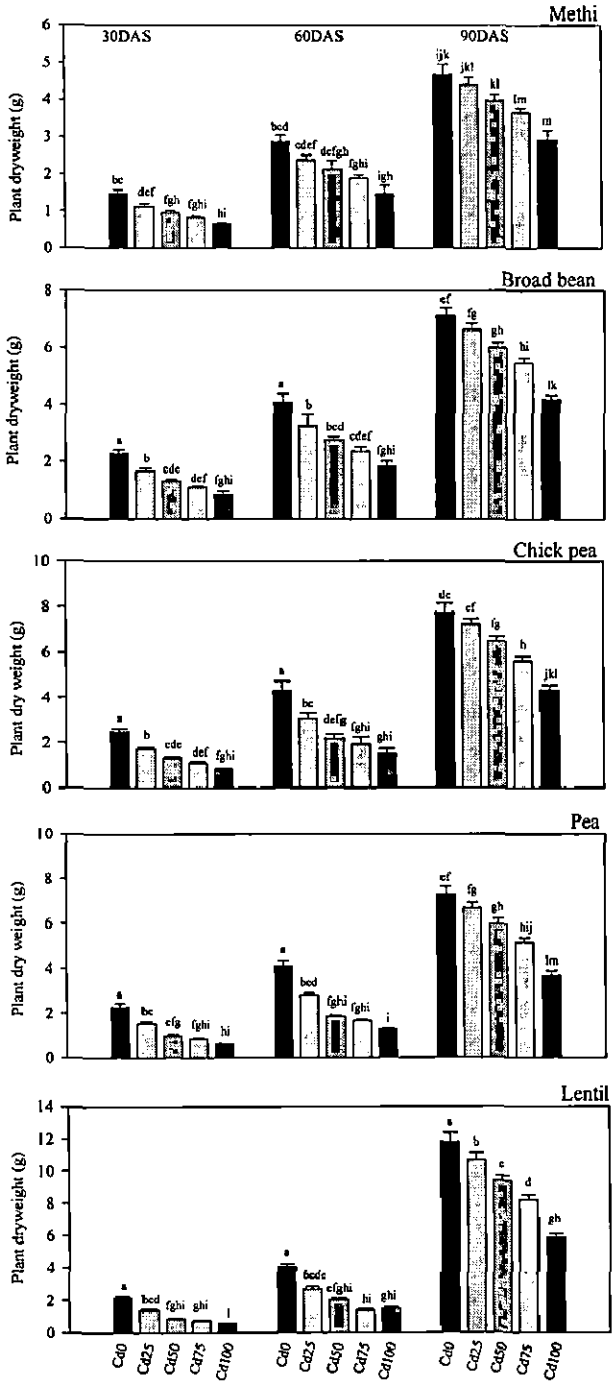


Figure 4.3: Effect of cadmium chloride ($CdCl_2$: 0, 25, 50, 75 and 100 mg Kg^{-1}) on the plant dry weight (g) of five legume crops at 30, 60 and 90 days after sowing.

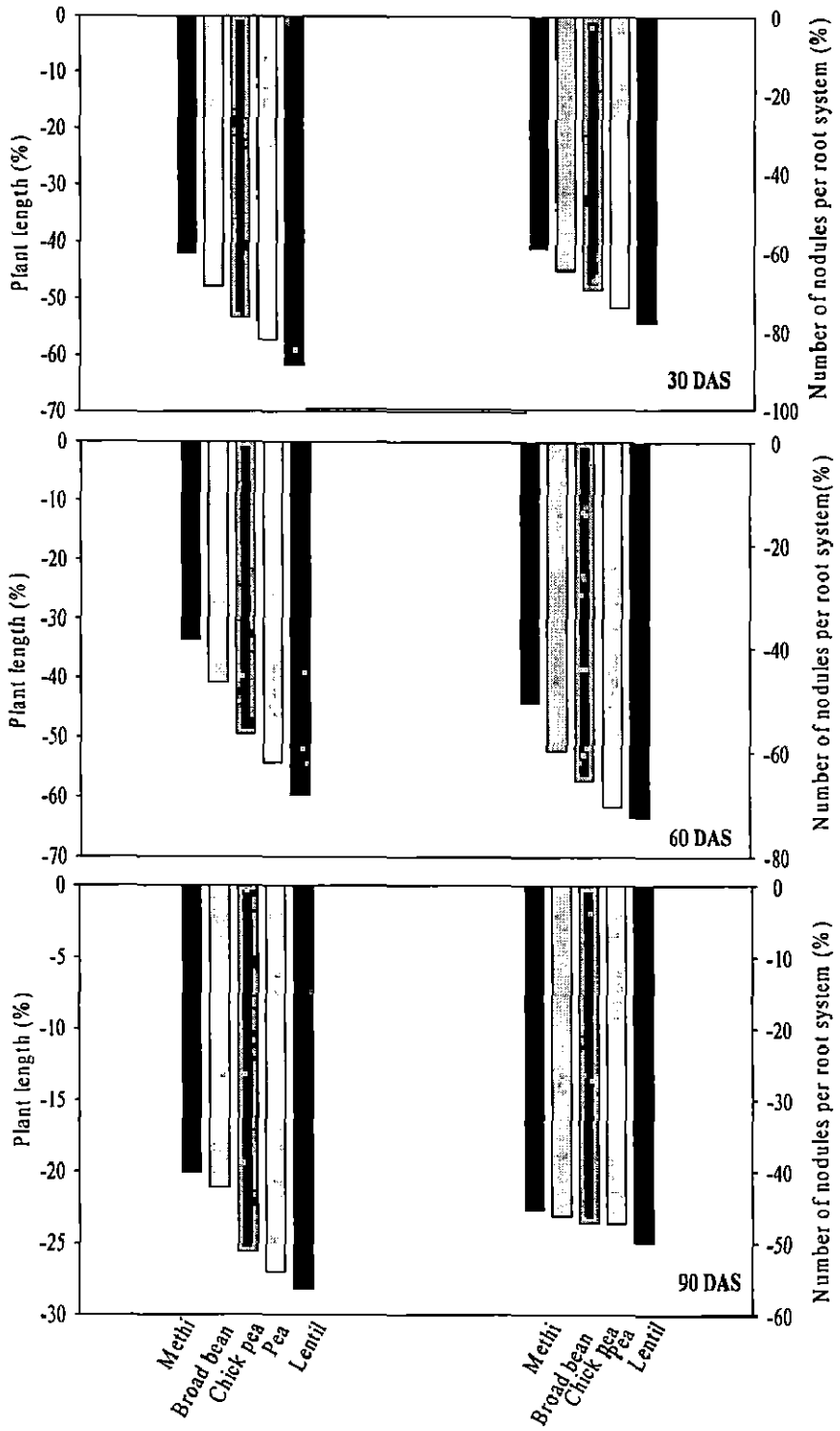


Figure 4.4: Per cent change in plant length and number of nodules per root system (%) of five legumes due to 100 mg Cd Kg^{-1} soil over control at 30, 60 and 90 days after sowing.

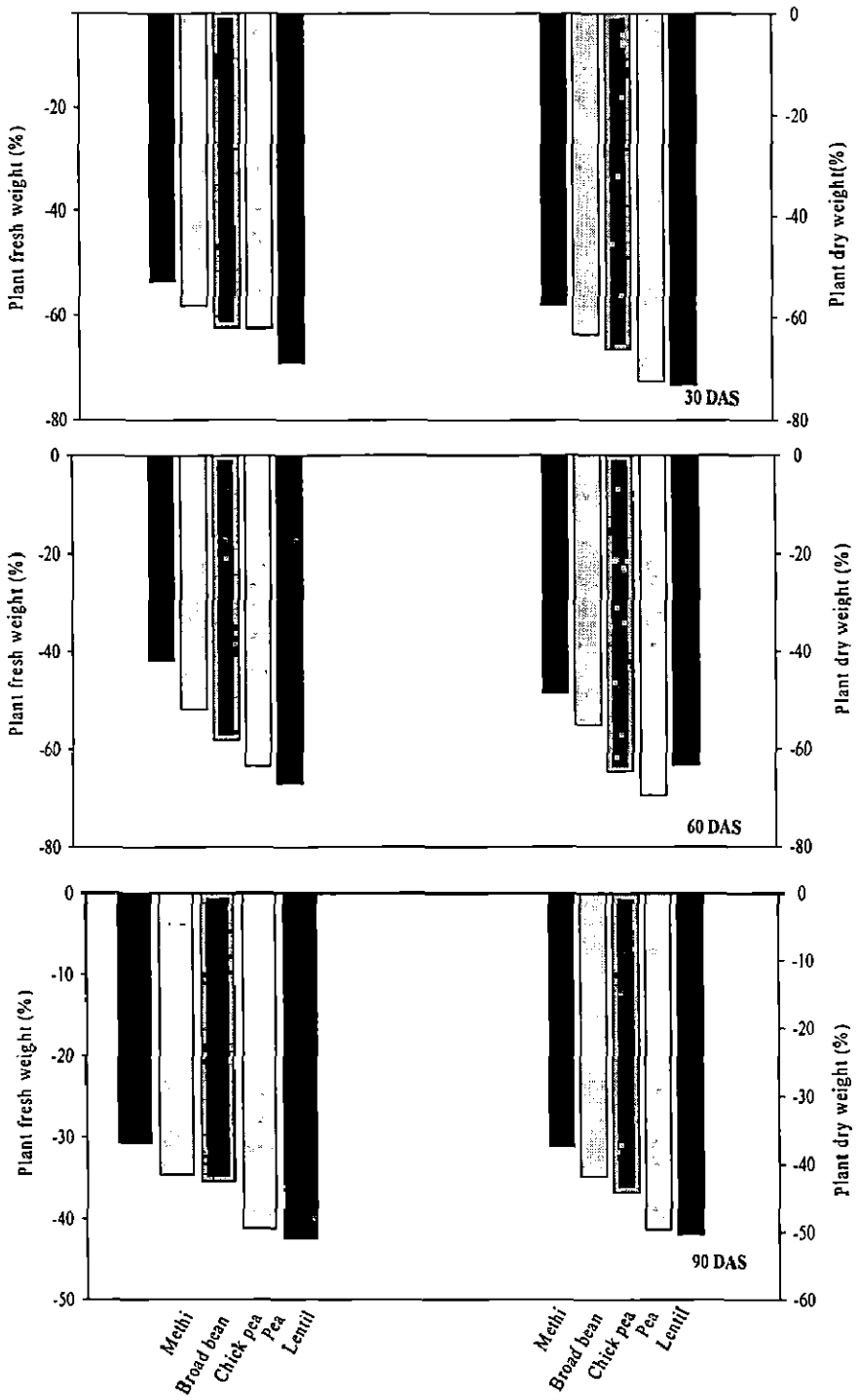


Figure 4.5: Per cent reduction in plant fresh weight and plant dry weight of five legumes due to 100 mg Cd Kg^{-1} soil over control at 30, 60 and 90 days after sowing.

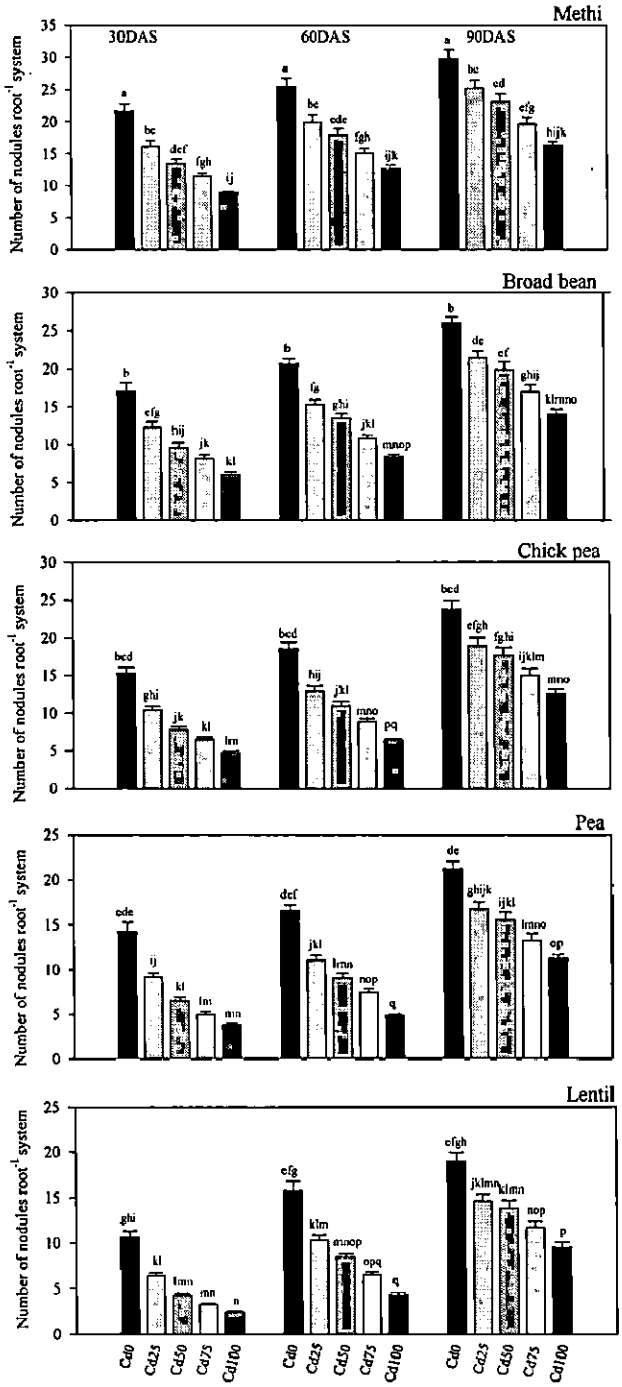


Figure 4.6: Effect of cadmium chloride ($CdCl_2$: 0, 25, 50, 75 and 100 mg Kg^{-1}) on the number of nodules per root system of five legume crops at 30, 60 and 90 days after sowing.

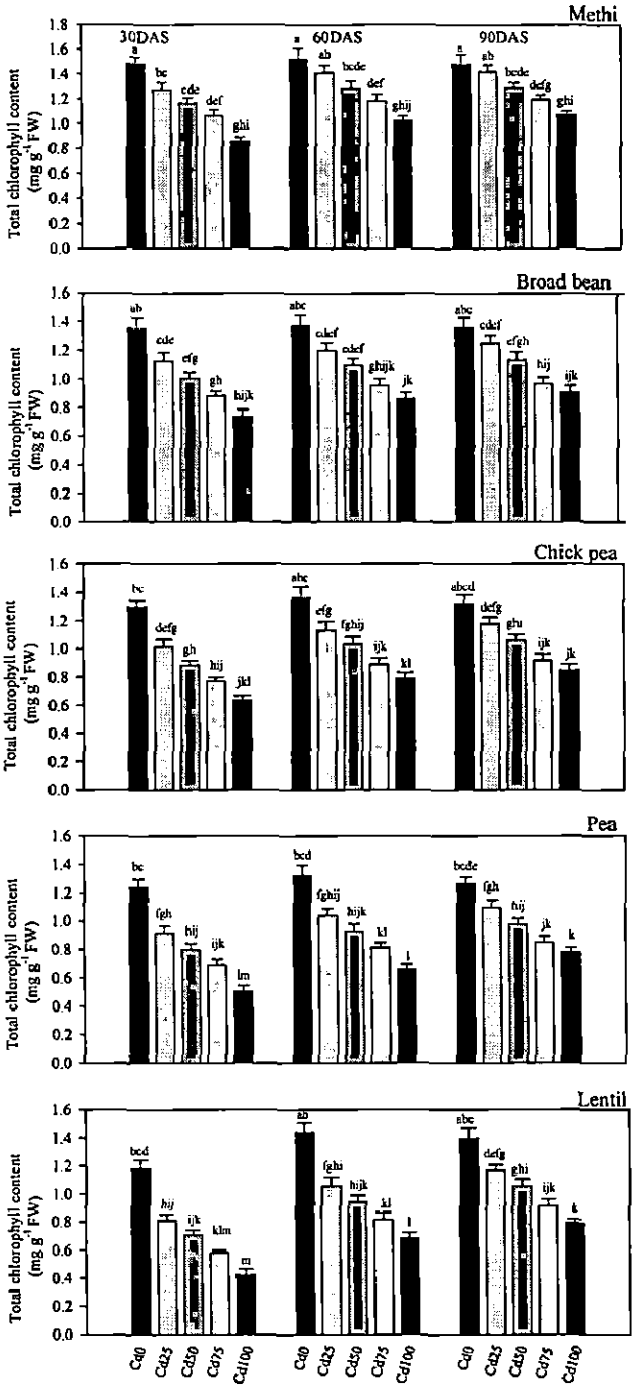


Figure 4.7: Effect of cadmium chloride ($CdCl_2$: 0, 25, 50, 75 and 100 mg Kg^{-1}) on the total chlorophyll (mg g^{-1} FW) content of five legume crops at 30, 60 and 90 days after sowing.

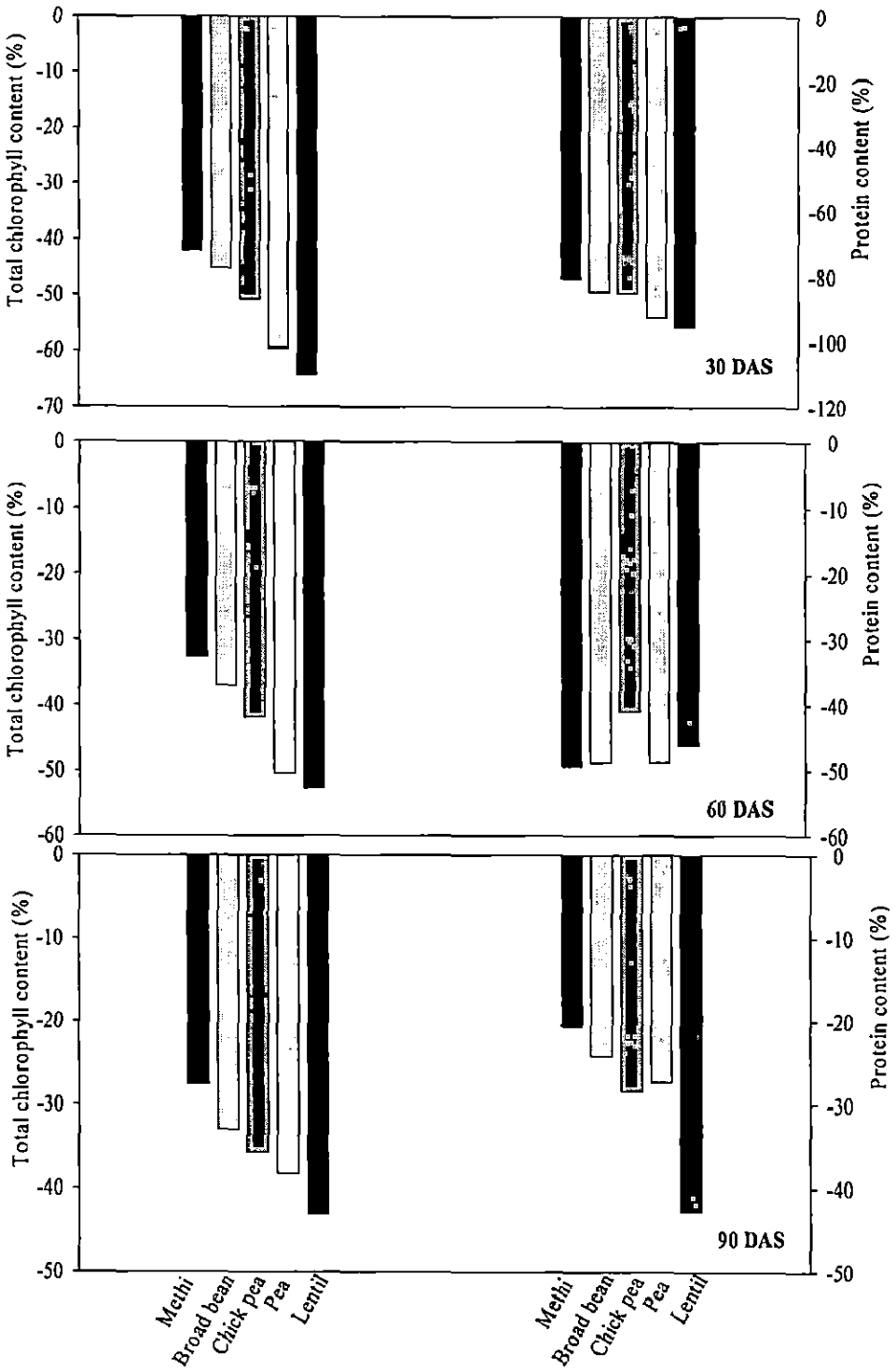


Figure 4.8: Per cent change in total chlorophyll and protein contents of five legumes due to 100 mg Cd Kg^{-1} soil over control at 30, 60 and 90 days after sowing.

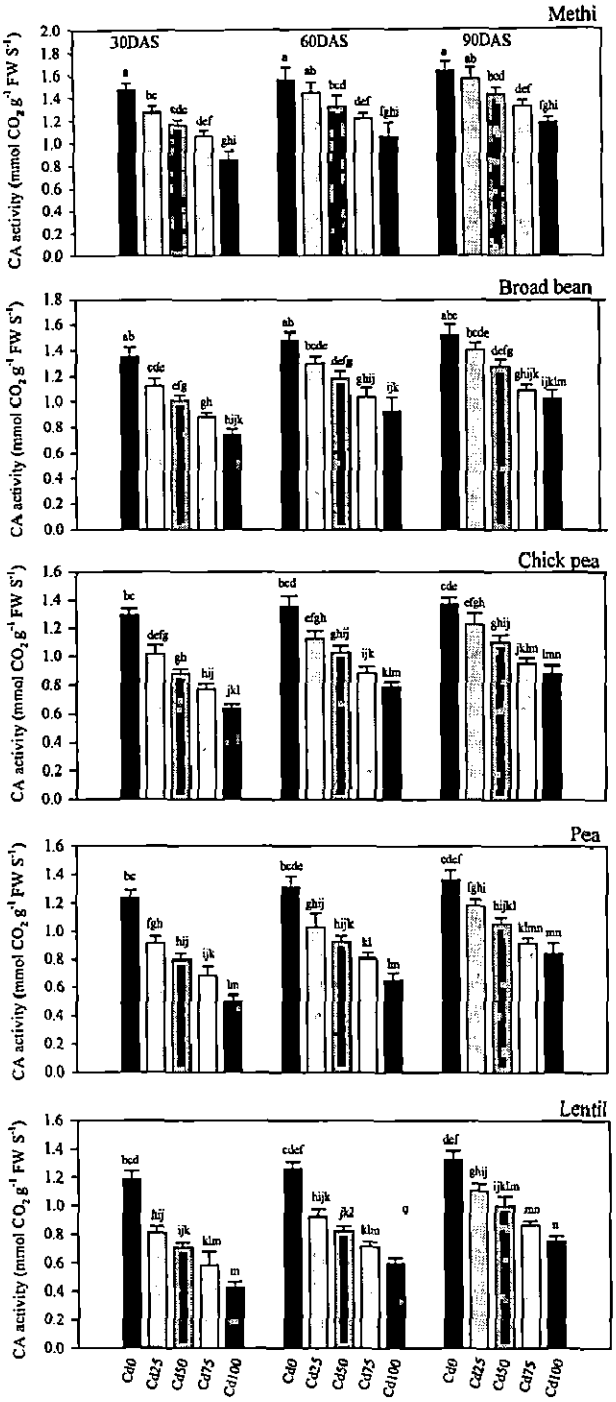


Figure 4.9: Effect of cadmium chloride (CdCl2: 0, 25, 50, 75 and 100 mg Kg^{-1}) on the carbonic anhydrase (mol CO_2 g^{-1}FM s^{-1}) activity of five legume crops at 30, 60 and 90 days after sowing.

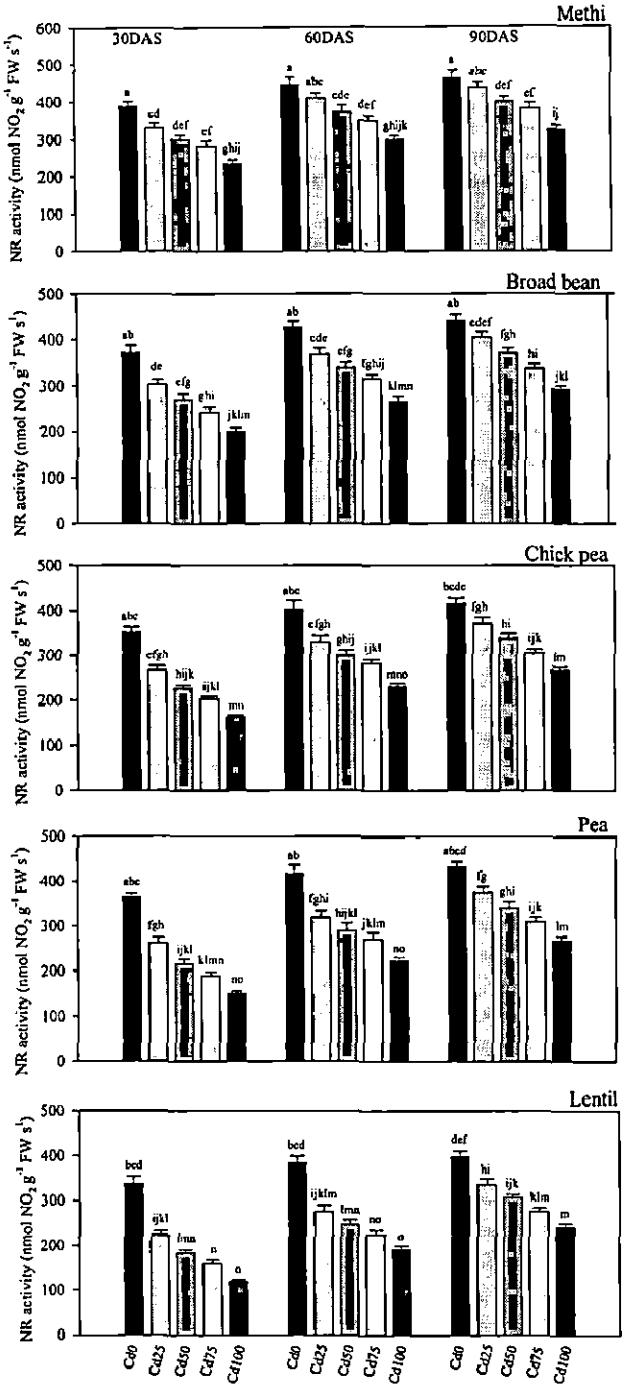


Figure 4.10: Effect of cadmium chloride ($CdCl_2$: 0, 25, 50, 75 and 100 mg Kg^{-1}) on the nitrate reductase (nmol NO_2 $g^{-1}FM$ s^{-1}) activity of five legume crops at 30, 60 and 90 days after sowing.

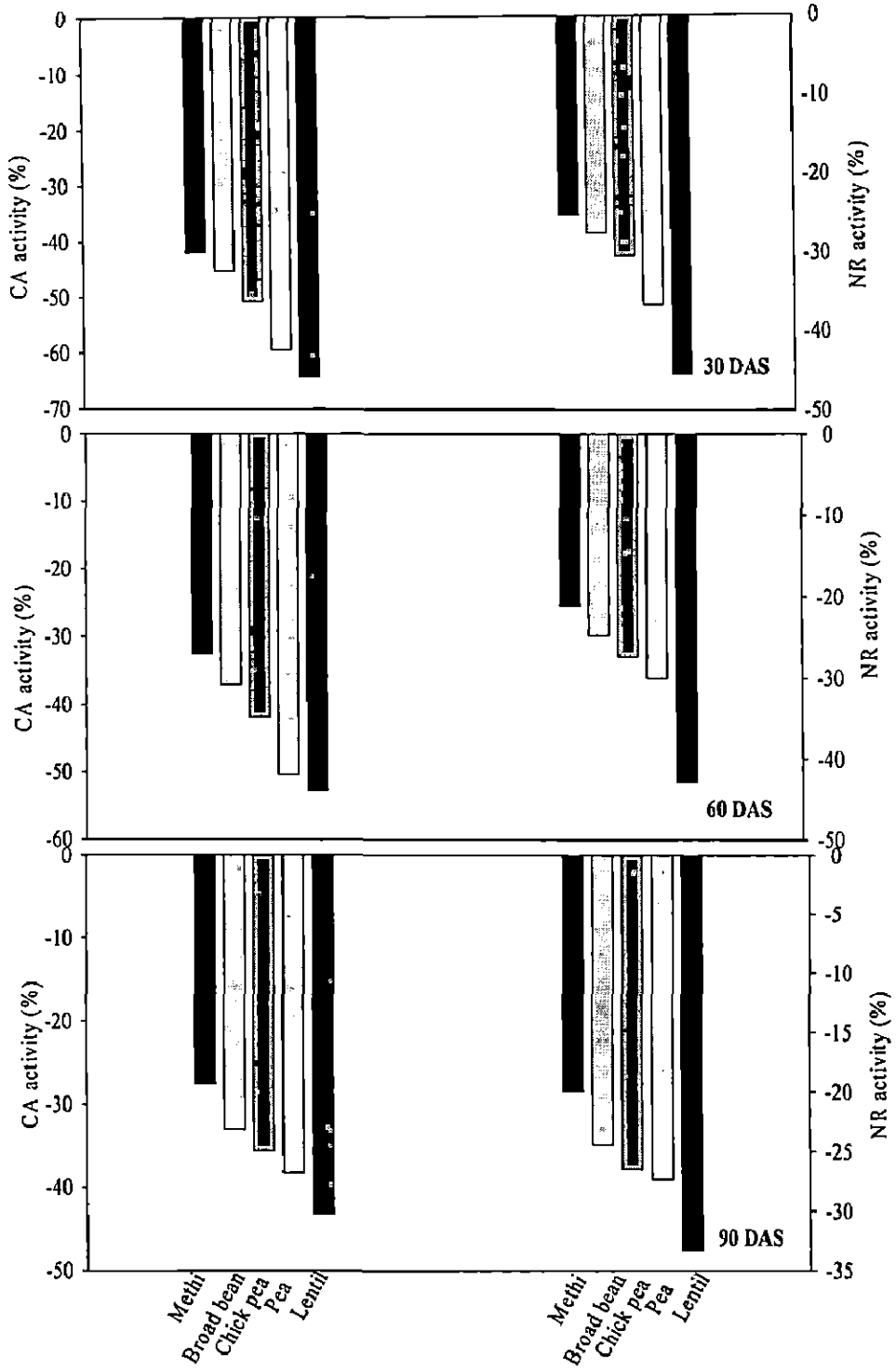


Figure 4.11: Per cent change in carbonic anhydrase and nitrate reductase activities of five legumes due to 100 mg Cd Kg^{-1} soil over control at harvest i.e., 120 days after sowing.

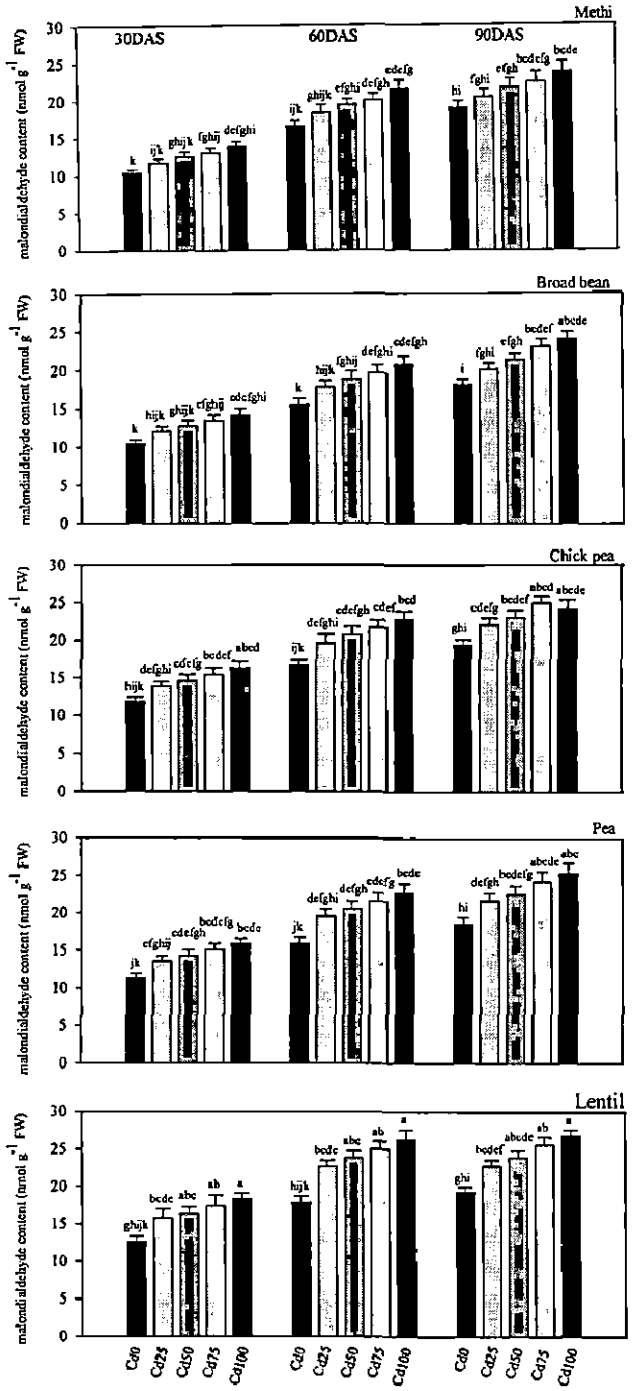


Figure 4.12: Effect of cadmium chloride ($CdCl_2$: 0, 25, 50, 75 and 100 mg Kg^{-1}) on the malondialdehyde (nmol g^{-1} FW) levelof five legume crops at 30, 60 and 90 days after sowing.

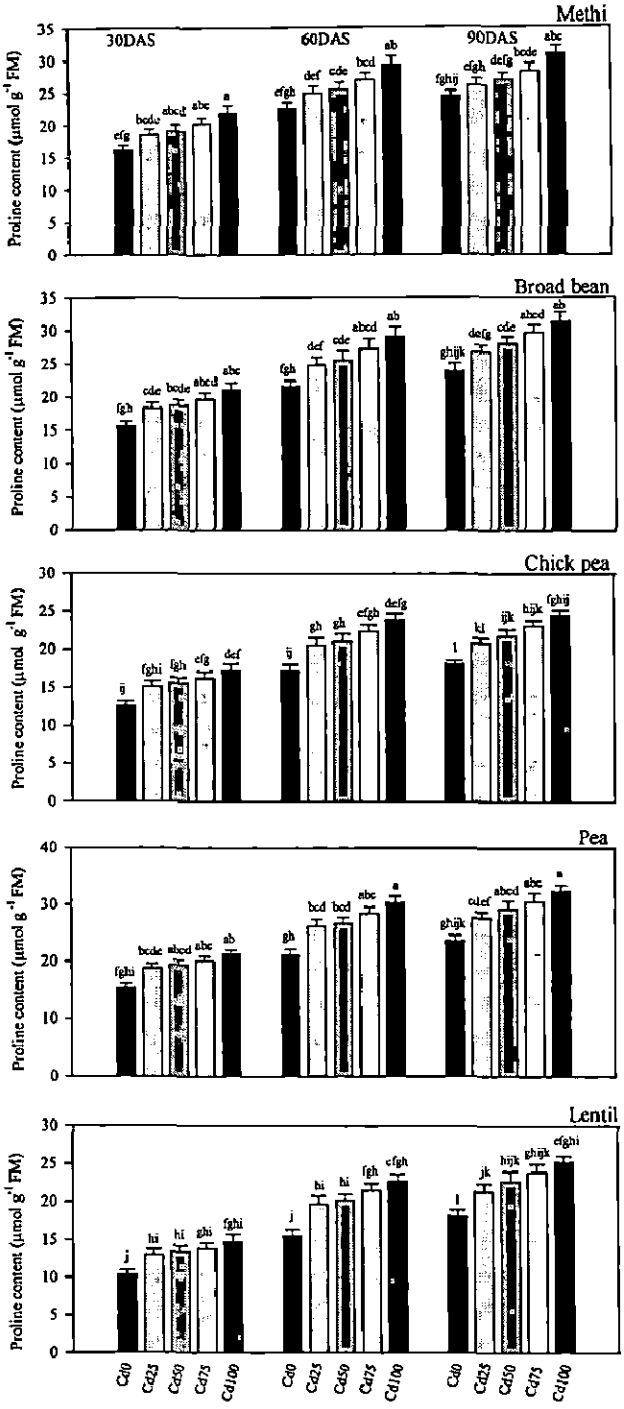


Figure 4.13: Effect of cadmium chloride ($CdCl_2$: 0, 25, 50, 75 and 100 mg Kg^{-1}) on the proline (µmol g^{-1} FM) content of five legume crops at 30, 60 and 90 days after sowing.

4.1.4 Carbonic anhydrase and nitrate reductase activities

Carbonic anhydrase (CA; E.C.4.2.1.1) and nitrate reductase (NR; E.C. 1.6.6.1) are the key enzymes regulating primary metabolism which decreased significantly with the treatments of legumes with Cd doses in soil (Figures 4.9 - 4.10). Maximum activity of CA was recorded in methi while minimum in lentil irrespective of the treatments. Activity of enzymes decreased with the increasing doses of Cd, whereas, it increased with the age of plants (Figure 4.11). CA showed the highest decline of 45.5% in lentil due to 100 mg Cd Kg^{-1} soil and lowest decline of 1.94 %, in methi due to 25 mg Cd Kg^{-1} soil.

The reduction in NR activity followed the same pattern to that of CA activity. However, the per cent reduction in its activity was more compared to CA irrespective of the treatments and growth stages (Figure 4.10). The tested legumes followed the order of reduction of NR activity as lentil> pea> chick pea> broad bean> methi (Figure 4.11). Methi and lentil showed a reduction in the activity of NR by 39.7, 64.8; 33.2, 50.3; and 29.8, 39.4% at 30, 60 and 90 DAS respectively, over its respective control due to 100 mg Cd Kg^{-1} soil.

4.1.5 Leaf protein content

Protein content decreased with the increase in dose of Cd (Figure 4.14). Lentil showed a decline of 25.9, 67.8, 83.2 and 95.3% at 30 DAS due to 0, 25, 50, 75 or 100 mg Kg^{-1} soil respectively, over the control. The pattern of increase in protein content was lentil< pea< chickpea< broad bean< methi. The per cent decrease in protein content was highest at 90 DAS (Figure 4.8) methi showed a decrease of 15.3, 40.0, 65.1 and 80.6 % due to 0, 25, 50, 75 and 100 mg Kg^{-1} soil respectively, over the control at 30 DAS.

4.1.6 Cd accumulation in plants

Cadmium accumulation increased in all the plants with the increasing doses of Cd in soil and also its per cent accumulation increased with the age of the plants (Figure 4.15). The trend of Cd accumulation in five legumes was lentil> pea> chick pea> broad bean> methi at all of the growth stages (Figure 4.16). Methi showed least accumulation of Cd whereas lentil showed its highest accumulation. In methi and lentil the accumulation of Cd was 0.9, 1.5; 1.1, 1.7; 1.3, 0.2 and 1.6, 2.4 μg g^{-1} dry

weight due to 25, 50, 75 or 100 Cd mg Kg^{-1} soil respectively, over the control at 90 DAS.

4.1.7 Yield characteristics

The increasing doses of Cd significantly decreased the yield characteristics (pod length, number of pods per plant, number of seeds per pod, weight of 1000 seeds and seed yield) of all the tested leguminous plants (Figure 4.17). Maximum reduction in yield characteristics was noted with 100 mg Cd Kg^{-1} soil. Lentil and pea exhibited similar and the greatest decrease in all yield attributes, whereas, methi showed lowest decrease followed by broad bean and chick pea. The better performance of legumes for yield characteristics was represented in order of methi> broad bean> chick pea> pea> lentil (Figure 4.18). Seed yield showed a reduction 53.6, 57.1, 59.8, 63.7 and 67.3% due to 100 Cd mg Kg^{-1} soil in methi, broad bean, chick pea, pea, lentil respectively, over the control at harvest.

4.1.8 Tolerance index

The tolerance index of legumes was calculated in terms of decrease of yield with the increasing doses of Cd. Methi showed highest tolerance index followed by broad bean, chick pea, pea whereas lentil emerged as the least tolerant (Figure 4.19). The tolerance index was 57.5 and 73.3% due to 100 mg Cd Kg^{-1} of soil in methi and lentil respectively.

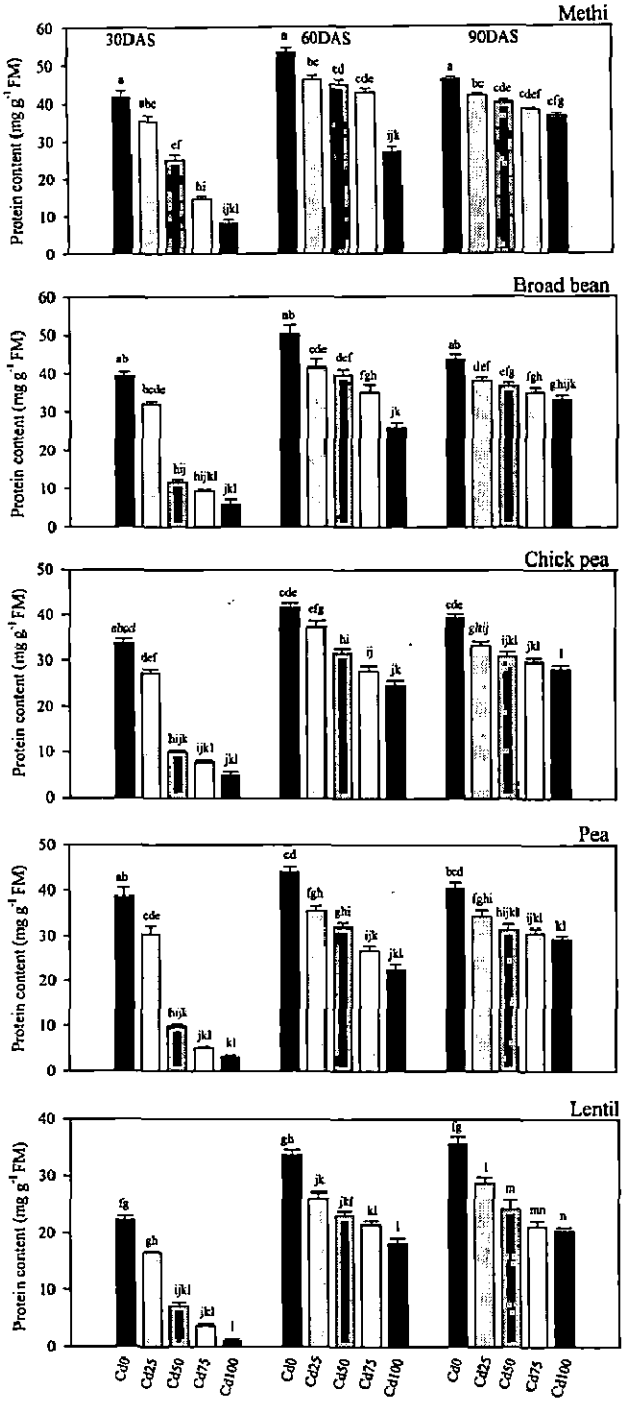


Figure 4.14: Effect of cadmium chloride ($CdCl_2$: 0, 25, 50, 75 and 100 mg Kg^{-1}) on the protein (mg g^{-1} FW) content five legume crops at 30, 60 and 90 days after sowing.

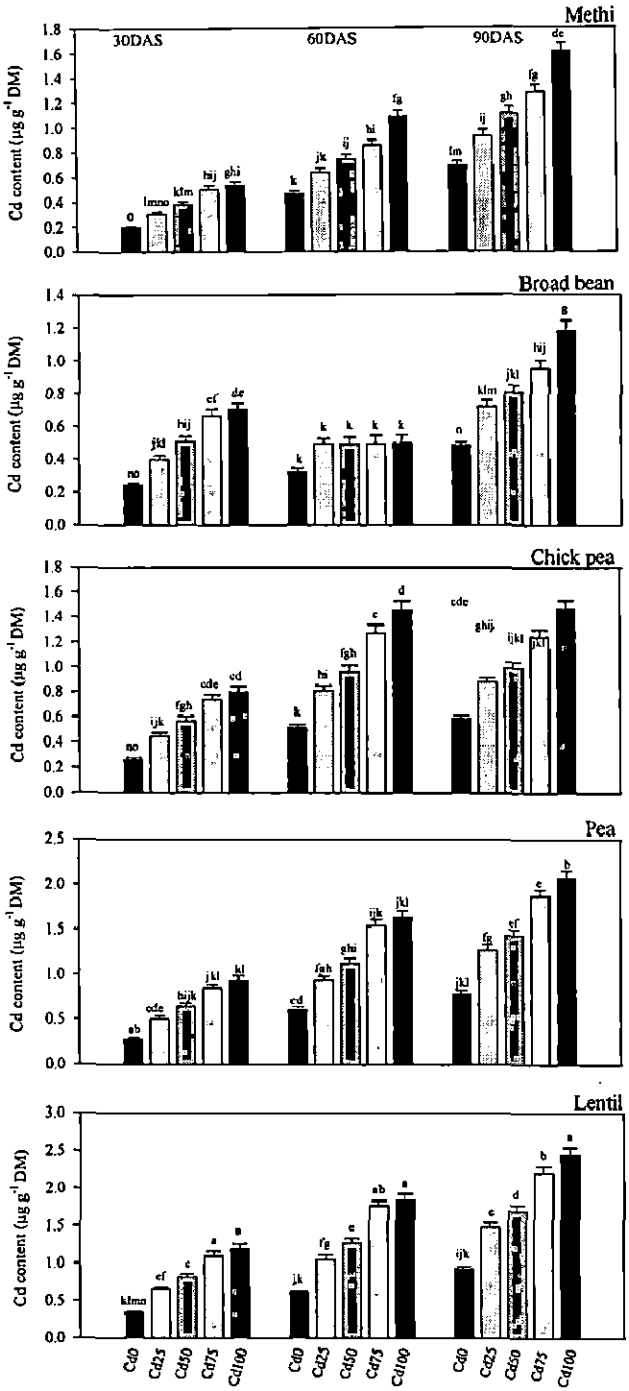


Figure 4.15: Effect of cadmium chloride ($CdCl_2$: 0, 25, 50, 75 and 100 mg Kg^{-1}) on plant cadmium (μg^{-1} DM) content of five legume crops at 30, 60 and 90 days after sowing.

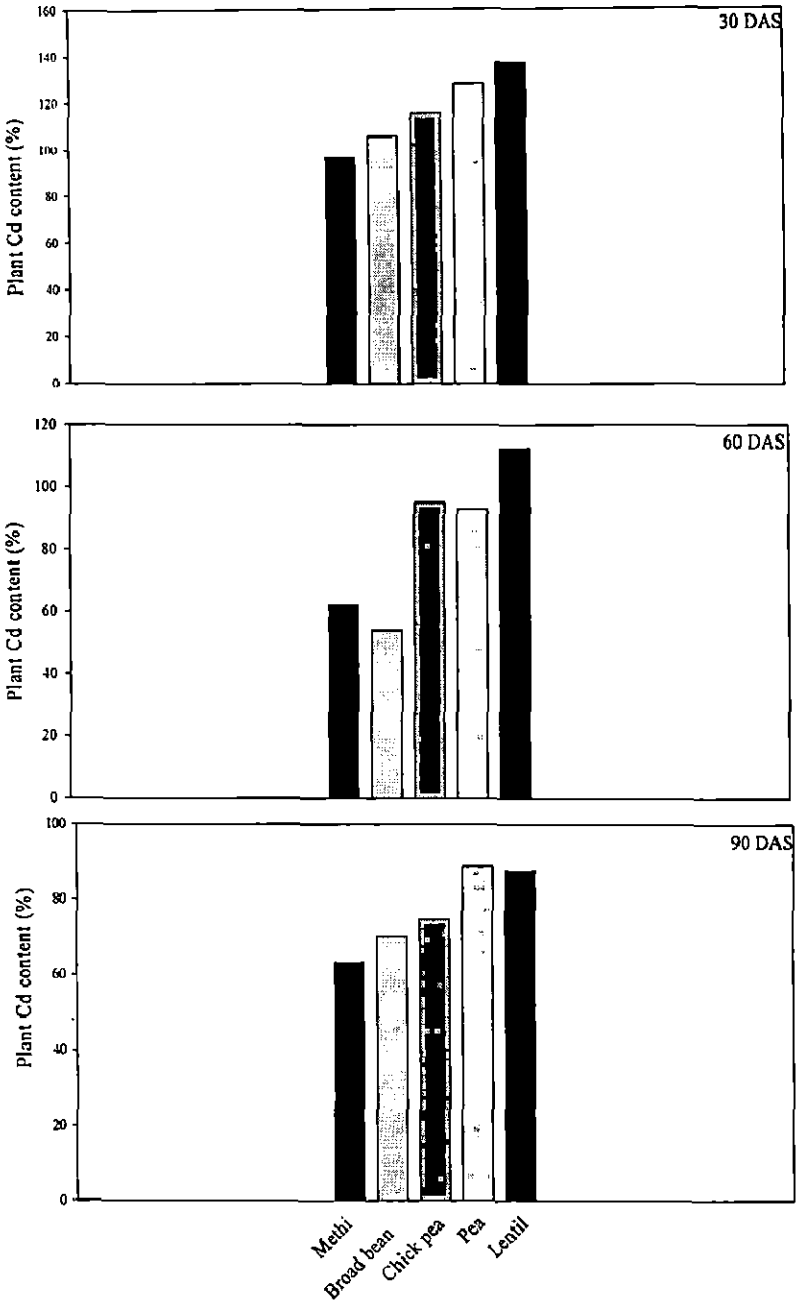


Figure 4.16: Per cent change in plant Cd content of five legumes due to 100 mg Cd Kg^{-1}soil over control at 30, 60 and 90 days after sowing.

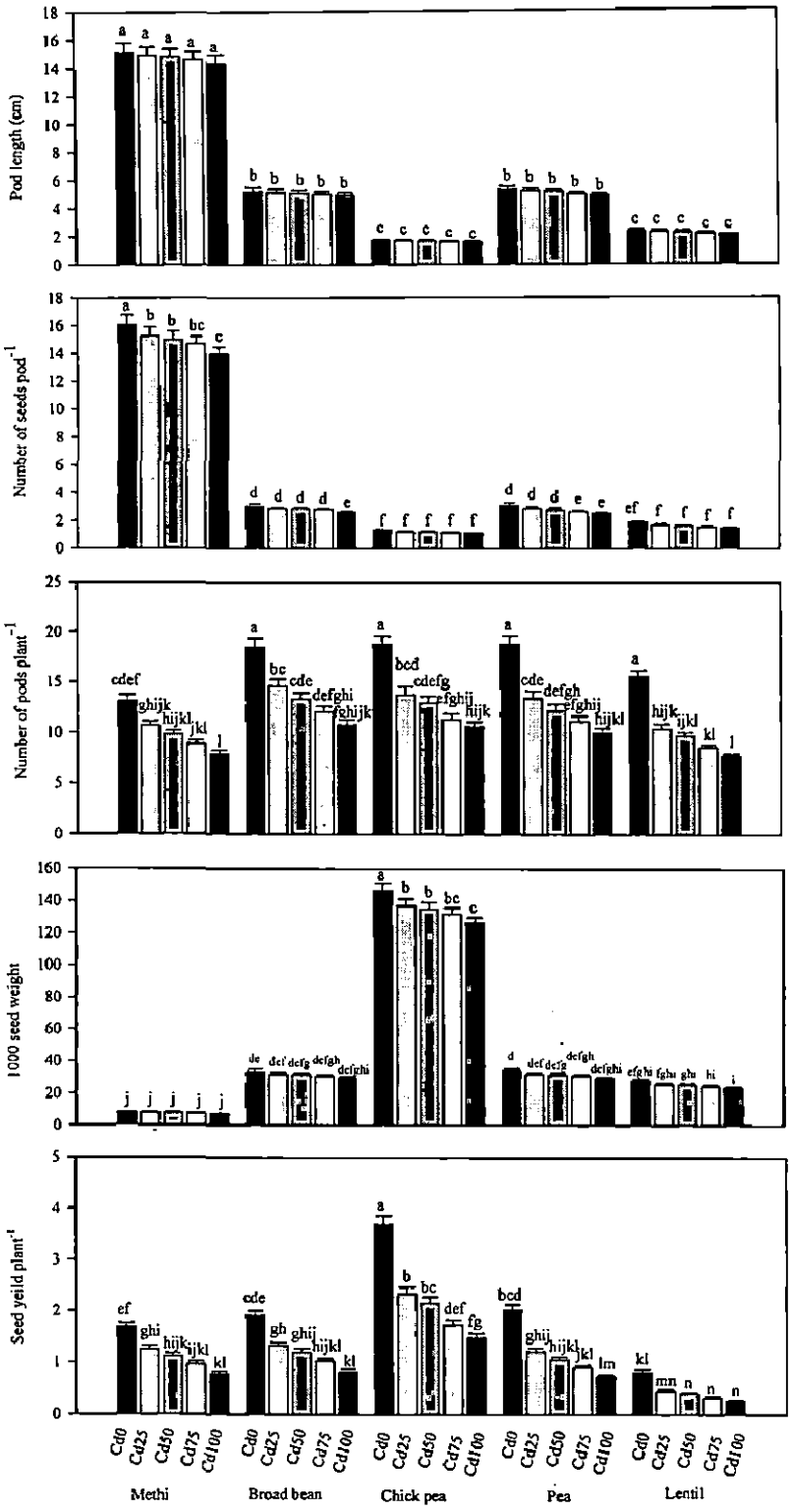


Figure 4.17: Effect of cadmium chloride ($CdCl_2$: 0, 25, 50, 75 and 100 mg Kg^{-1}) on pod length (cm), number of pods $plant^{-1}$, number of seeds pod^{-1}, 1000 seeds weight and seed yield $plant^{-1}$ of five legume crops at harvest i.e., 120 days after sowing.

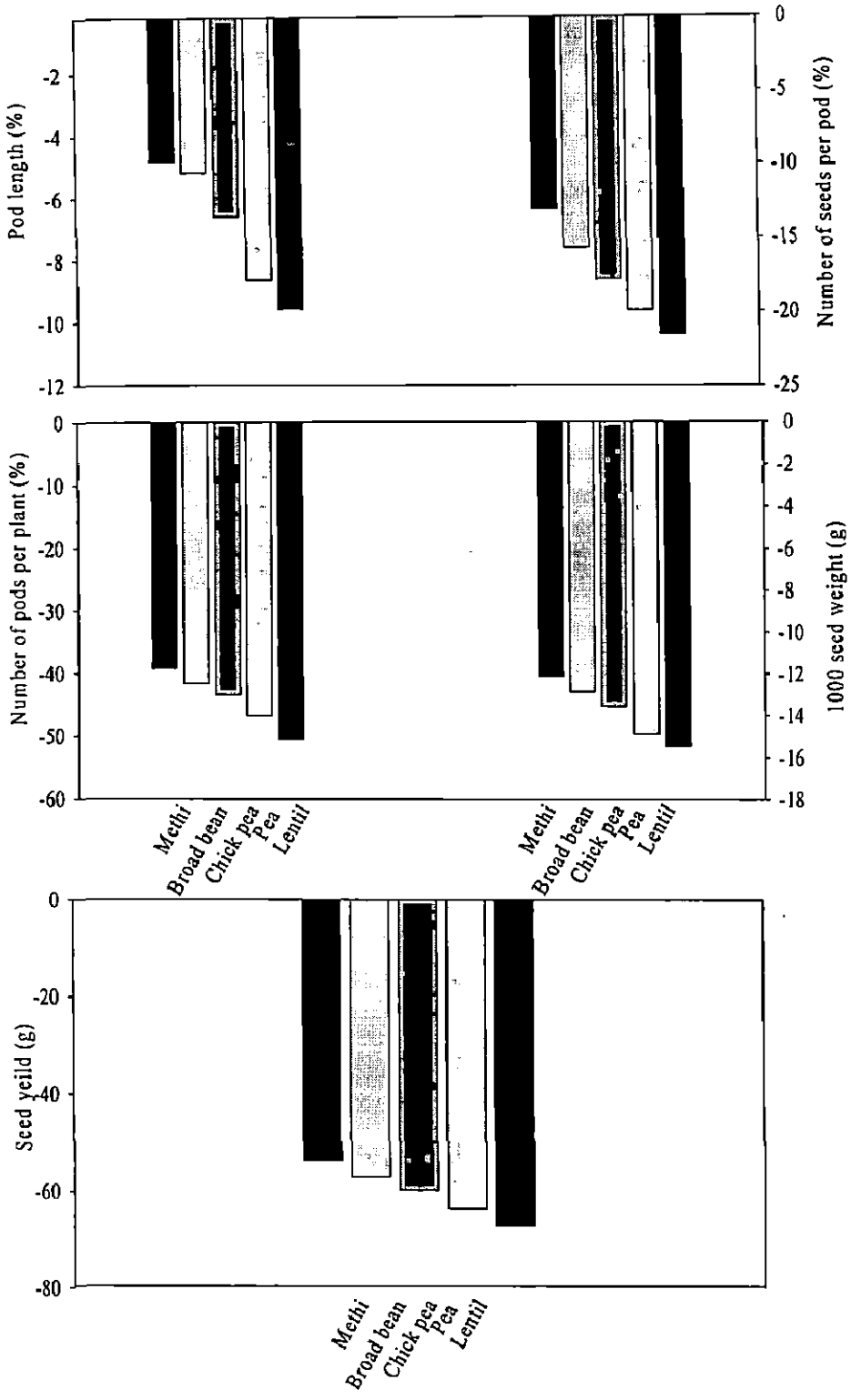


Figure 4.18: Per cent change in pod length, number of seeds per pod(%), number of pods per plant (%), 1000 seed weight (g) and seed yield (g) of five legumes due to 100 mg Cd Kg^{-1} soil over control at 30, 60 and 90 days after sowing.

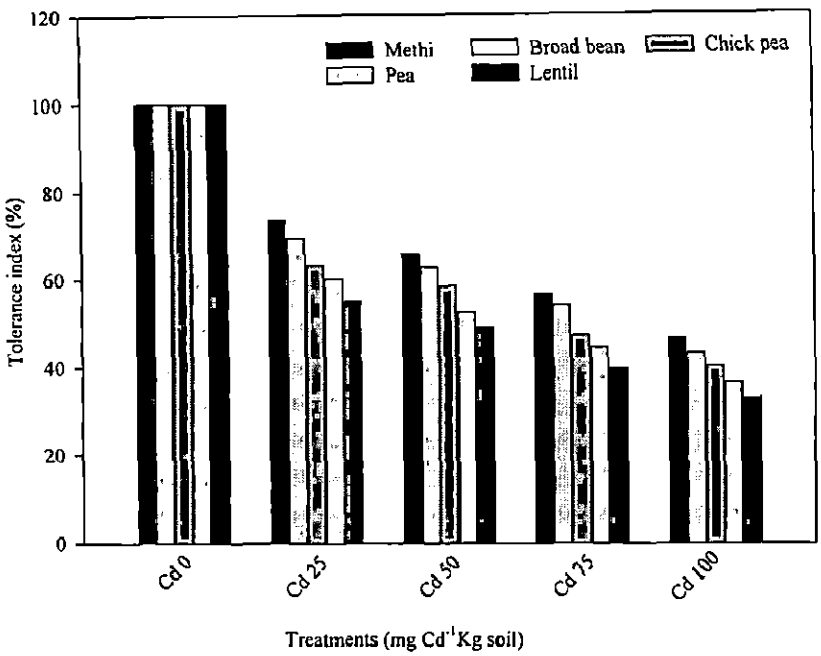


Figure 4.19: Tolerance index of five legumes (methi, broad bean, chickpea, pea, and lentil) exposed to 0, 25, 50, 75 and 100 mg Cd Kg^{-1} soil. Tolerance index was calculated as per cent change of seed yields with control.

Summary of Experiment 1

- The accumulation of Cd in plants was found in Cd dose dependent which was maximum in lentil followed by methi, broad bean, chick pea, pea, and lentil.
- Cadmium treatments resulted in an overall reduction in growth, biochemical and yield characteristics in all plants with more pronounced decrease with 100 mg Cd Kg^{-1} soil treatment.
- Among legumes, lentil showed greater reduction in growth, biochemical and yield characteristics whereas, least decrease of these characteristics was found in methi.
- The stress markers (MDA and proline content) increased with the increasing doses of Cd with maximum values observed in lentil and minimum in methi at all the growth stages.
- The trend of sensitivity of leguminous plants to Cd toxicity was as follows lentil> pea> chick pea> broad bean> methi.
- On the basis of overall performance of the legumes under Cd stress, methi emerged as Cd least-sensitive and lentil as Cd most-sensitive.

4.2 Experiment 2: To study the alleviation potential of *Rhizobium* inoculation against Cd-induced effects in Cd sensitive and Cd non-sensitive legumes

Experiment 2 was conducted on the basis of the findings of Experiment 1. As observed in Experiment 1, methi emerged as least Cd-sensitive and lentil as most Cd - sensitive legume. The treatment 100 mg Cd Kg^{-1} soil caused maximum decrease in the observed characteristics and 50 mg Cd Kg^{-1} soil was moderate toxic in effects. Therefore, the present experiment, was aimed to study the alleviation potential of *Rhizobium* under the moderate (50 mg Cd Kg^{-1} soil) and the highest (100 mg Cd Kg^{-1}soil) Cd levels by studying the changes in Cd accumulation in root and shoot, growth, biochemical characteristics, stress markers, components of antioxidant systems and yield attributes.

The details of results briefly described below and summarized in Figures (4.20 – 4.34)

4.2.1 Growth characteristics

Both the legumes significantly differed in growth characteristics (shoot length, root length, fresh and dry weight of root and shoot, number and area of leaves per plant and nodules number per root system) decreased in both the plants treated with Cd and the was proportional to the level of Cd (50 mg Cd Kg^{-1} soil and 100 mg Cd Kg^{-1}soil) plant at all the stages of growth, however, treatment of *Rhizobium* nullified effects of 50 mg Cd Kg^{-1} soil, but could not completely ameliorate the effects 100 mg Cd Kg^{-1}soil (Figures 4.20 - 4.24). Lentil showed higher decrease in growth characteristics than methi and the effect of Cd was more pronounced at early growth stage (30 DAS) than at later stage (90 DAS). Methi and lentil showed a decrease of 37.9, 57.4% in shoot length; 22.3, 28.5% in root length; 45.8, 86.9% in shoot fresh weight; 15.2, 25.8% in root fresh weight; 54.7, 39.7% in shoot dry weight; 16.3, 27.7% in root dry weight respectively, over the control due to 50 mg Cd Kg^{-1} soil at 30 DAS. Shoot length, shoot fresh and dry weights decresed due to 100 mg Cd Kg^{-1} soil. Seeds inoculated with *Rhizobium* caused maximum enhancement in the observed growth characteristics in control plants. In methi, an increase of 89.6, 58.5% in shoot and root length; 53.1, 41.6% in fresh weight of shoot and root; 32.5, 43.3%, in dry weight of shoot and root; 67.5 and 70.2% in leaf number and area due to *Rhizobium* inoculation in control plants at 90 DAS. In methi, an increase of 39.6, 37.2% in shoot and root

length; 17.9, 19.7% root fresh weight and dry weight; 91.9, 24.7% in leaf number and leaf area and 30.1% in number of nodules respectively, over the control due to 50 mg Cd Kg^{-1} soil at 90 DAS. The extent of increase in growth was more in plants supplemented with 50 mg Cd Kg^{-1} soil compared to 100 mg Cd Kg^{-1} soil.

4.2.2 Photosynthetic attributes

The photosynthetic parameters (chlorophyll a, chlorophyll b, total chlorophyll and carotenoids content) decreased with the increase in the level of Cd in soil at all growth stages (Figures 4.25 - 4.26). However, the content of pigment in the two legumes was insignificantly different irrespective of the treatments or age. Cadmium amended in soil significantly declines these parameters as compared to control but this decrease was more due to 100 mg Cd Kg^{-1} of soil. Chlorophyll a, chlorophyll b, total chlorophyll and carotenoid content showed a decrease of 29.0, 54.0; 14.6, 26.7; 24.8, 46.2; 30.8 and 46.2% in methi and lentil respectively, over the control at 30 DAS due to 50 Cd mg Kg^{-1} of soil. *Rhizobium* completely alleviated Cd stress improved the pigment contents. Chlorophyll a, chlorophyll b, total chlorophyll and carotenoid content showed an increase of 29.4, 26.9; 27.6, 24.7; 28.9, 24.7 and 60.1, 52.7% in methi and lentil respectively, compared to control due to 50 Cd mg Kg^{-1} of soil at 90 DAS

4.2.3 Metabolic stress markers: Lipid peroxidation and proline content

The lipid peroxidation and proline contents have shown gradual increase with the age from 30 to 90 DAS (Figure 4.27). The proline content was higher in methi than lentil, whereas, MDA (malondialdehyde) content was higher in lentil than methi. Lipid peroxidation increased and proline content decreased with the increase in the level of Cd in soil at all growth stages. The highest proline and MDA contents were 53.8, 41.7% and 38.4, 53.7% in methi and lentil respectively, over the control due to 100 mg Cd Kg^{-1} soil, at 30 DAS. Legumes inoculated with Inoculation of *Rhizobium* showed significant increase in proline content in methi at 30 and 60 DAS. Legumes inoculated with *Rhizobium* showed non-significant decrease in MDA and proline content. However, this decrease was more in plants supplemented with 50 mg Cd Kg^{-1} of soil. This symbiont MDA and proline content showed a decrease of 21.1, 29.4 and 28.1, 29.4% in methi and lentil respectively, over the control at 30 DAS.

4.2.4 Enzymatic stress markers: Antioxidant activity

Activity of antioxidant enzymes increased as the age of two legumes progressed from 30 to 90 DAS (Figures 4.28 - 4.29). An elevation of 47.4, 38.9% in POX; 47.4, 37.8% in CAT and 50.7, 44.5% in SOD activity were recorded in methi and lentil respectively, over the control in plants supplemented with 100 mg Cd Kg^{-1} soil at 30 DAS. Inoculation with *Rhizobium* enhanced the activity of these enzymes. However, POX and SOD increased significantly in both the legumes at all the stages but CAT increased insignificantly compared to non-inoculated plants under Cd stress. The activity of POX, CAT and SOD showed an increase of by 38.8, 34.2; 42.7, 37.5and 35.9, 54.3% in methi and lentil respectively, with respect to control plants at 30 DAS, when plants were inoculated with *Rhizobium* and supplemented with 100 mg Cd Kg^{-1} soil.

4.2.5 Carbonic anhydrase and nitrate reductase activities

Carbonic anhydrase and NR are the key enzymes of primary metabolism. These enzymes exhibited higher activity in methi compared to lentil (Figure 4.30). Conversely, addition of Cd decreased the activity of both the enzymes. However, this decrease was more with 100 mg Cd Kg^{-1} soil. A decline of 23.5, 39.7% in CA and 23.0, 45.7% in NR activity was recorded in methi and lentil respectively, as compared to control due to 50 mg Cd Kg^{-1} soil at 30 DAS. Plants inoculated with *Rhizobium* showed elevated activity of these enzymes however, maximum activity was recorded in control plants. Among the two legumes, methi showed highest increase of 55.6 and 68.3% in CA and NR respectively, as compared to control. CA and NR activity in methi showed an increase of 16.7 and 54.9% respectively, compared to control plants due to inoculation of *Rhizobium* in plants supplemented with 50 mg Cd Kg^{-1} soil at 90 DAS.

4.2.6 Leaf protein content

The leaf protein content also followed the similar trend for treatments as that of the enzymes. However, it was comparatively higher in *Rhizobium* inoculated plants compared to non-inoculated plants supplemented with 50 or 100 mg Cd Kg^{-1} soil (Figure 4.31).The per cent increase in protein content of leaf was highest at 60 DAS as compared to early or later growth stages. Enzyme activity and protein content in methi reflected higher values than lentil. Addition of Cd decreased the protein

content. However, this decrease was more with 100 mg Cd Kg^{-1} soil. A decline of 57.9 and 64.2% in methi and lentil respectively, as compared to control due to 50 mg Cd Kg^{-1} soil at 30 DAS. Plants inoculated with *Rhizobium* showed increase of 59.9 and 51.8% in both the legumes due to 50 mg Cd Kg^{-1} soil at 60 DAS as compared to control.

4.2.7 N, P and K content in leaves

The leaf N, P and K content also followed the similar trend as that of protein and it decreased with the increase in the level of Cd in soil (Figures 4.31- 4.28). *Rhizobium,* increased the leaf N, P and K contents in Cd treated plants and this increase was maximum in control plants. Methi showed more accumulation of these macronutrients than lentil. Methi showed an increase of 35.5, 29.0 and 24.5% in N, P and K content respectively, in control plants inoculated with *Rhizobium* at 90 DAS.

4.2.8 Cadmium accumulation in shoot and root

Cadmium content in the shoot and root of both the legumes increased with the increasing dose of Cd in soil. Lentil accumulated more Cd in shoot and root than methi. The content of Cd accumulation was more in the shoot as compared to root in both the legumes. However, the Cd content increased with the age of legumes irrespective of the treatments (Figure 4.32). The application of *Rhizobium* to seeds maximally lowered the Cd content in the root and shoot of both the legumes treated with 50 mg Cd Kg^{-1} soil followed by 100 mg Cd Kg^{-1} soil treated plants. The minimum Cd accumulation was observed in control plants. In lentil, 100 mg Cd Kg^{-1} soil caused an accumulation 2.2μg Cd g^{-1} and 1.5μg Cd g^{-1} dry weight of the tissue of root and shoot respectively, at 90 DAS. However, *Rhizobium* inoculated plants in lentil gave the accumulation of 1.8μg^{-1} Cd g^{-1} in roots and 0.9 Cd g^{-1}in shoot.

4.2.9 Yield characteristics

Cadmium treatments decreased the yield parameters (pod length, number of pods per plant, number of seeds per pod, seed yield per plant and 1000 seed weight) of the two legumes at the time of harvest and the extent of decrease was more in lentil as compared to methi (Figure 4.33). Number of pods per plant and seed yield per plant showed significant decrease when Cd was supplemented to the soil but inoculation of *Rhizobium* partially recovered yield characteristics against both the Cd treatments. Pod number per plant and seed yield per plant showed a decrease of 24.8 and 19.5, 34.1, 28.0% respectively, due to *Rhizobium* inoculated plants treated with 50 mg Cd

Kg^{-1}soil. The inoculation of *Rhizobium* alone increased the number of pods per plant and seed yield by 22.2, 17.63 and 38.7, 28.0% in methi and lentil respectively, compared to control.

4.2.10 Tolerance index

The tolerance index of legumes was calculated in terms of decrease in yield with the increasing doses of Cd. Methi showed highest tolerance followed by broad bean, chick pea, pea whereas lentil emerged as the least tolerant. However, inoculation of *Rhizobium* showed an increase of 28.0, 40.9% in methi and 35.4, 48.4% in lentil due to 50 or 100 mg Cd Kg^{-1} of soil respectively, over the control.

Summary of Experiment 2

- Seeds inoculated with *Rhizobium* proved effective in alleviating the toxic effects of Cd and reduced the accumulation of Cd at all the growth stages. In general, the Cd content was higher in root than shoot irrespective of the legumes or the treatment.
- Application of *Rhizobium* proved effective in alleviating the effects of 50 mg Cd Kg^{-1} soil on growth, biochemical and yield characteristics of methi and lentil.
- Cadmium treatments down regulated nitrogen assimilaton pathway and decreased the nitrogen content and NR activity. Moreover, *Rhizobium* inoculation reduced the Cd stress and increased the activity of enzyme and nitrogen content.
- Cd-induced accumulation of lipid peroxidation (MDA level) was significantly lowered by *Rhizobium* inoculated seeds.
- Responses of antioxidant enzyme activities were found differential in both the legumes under Cd stress. *Rhizobium* inoculated seeds increased the activities of POX, CAT and SOD of both the doses 50 and 100 mg Cd Kg^{-1} soil.
- Inoculation of *Rhizobium* maximally lowered the Cd toxicity against 50 mg Kg^{-1} soil as reflected by decrease in malondialdehyde level and increased the proline content in both the legumes.
- Inoculation of *Rhizobium* to seeds lowered the Cd-induced decrease in yield characteristics of both the legumes particularly, number of pods and seed yield per plant.
- *Rhizobium* inoculation partially alleviated the Cd stress caused due to 100 mg Cd Kg^{-1} in both the legumes.

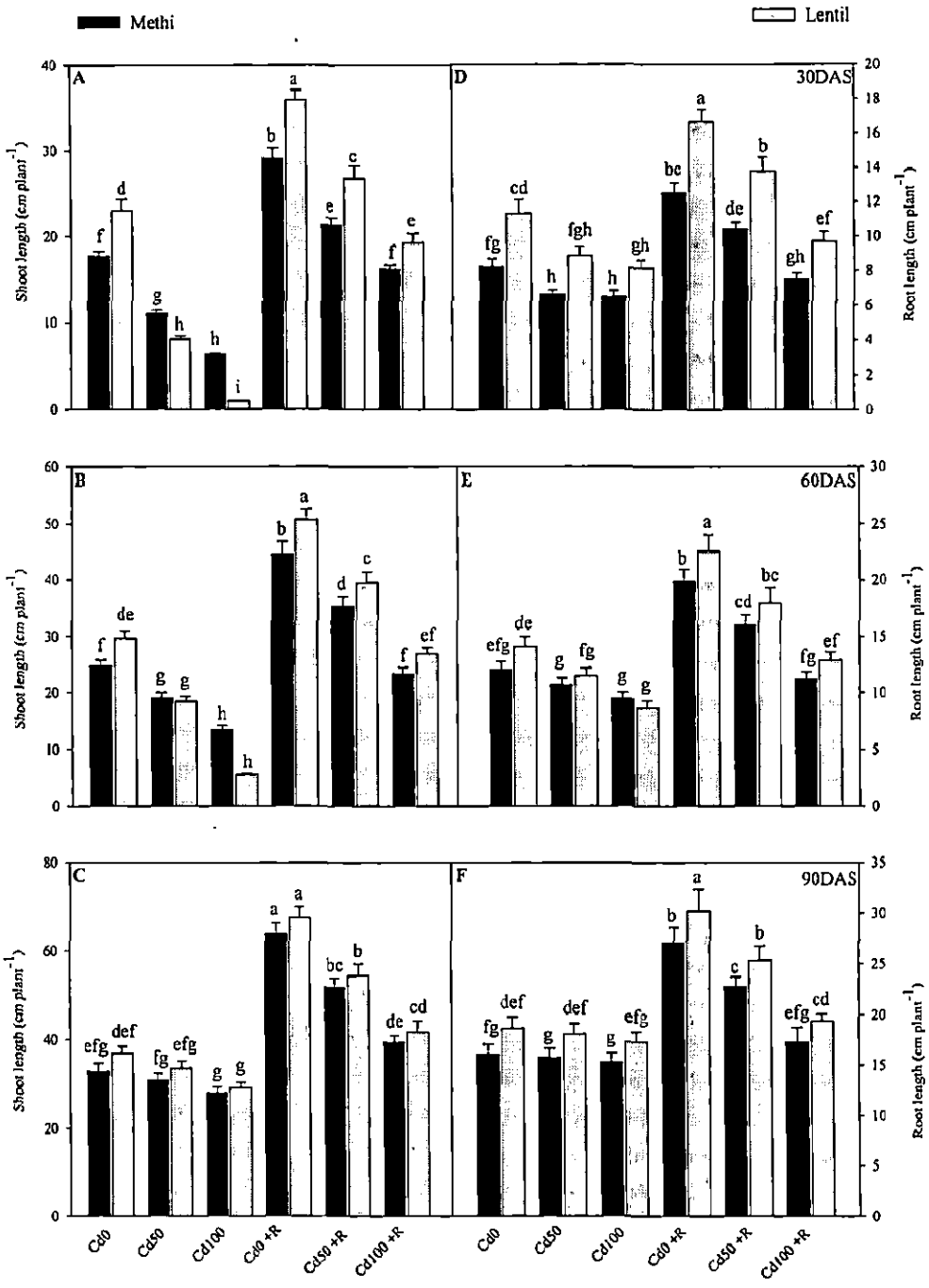


Figure 4.20: Effect of *Rhizobium* (R) inoculation against 0, 50 and 100 mg $CdCl_2$ Kg^{-1} soil on shoot length and root length (cm) of methi (*Trigonella foenum-graecum*) and lentil (*Lens culanaris*) at 30, 60 and 90 days after sowing.

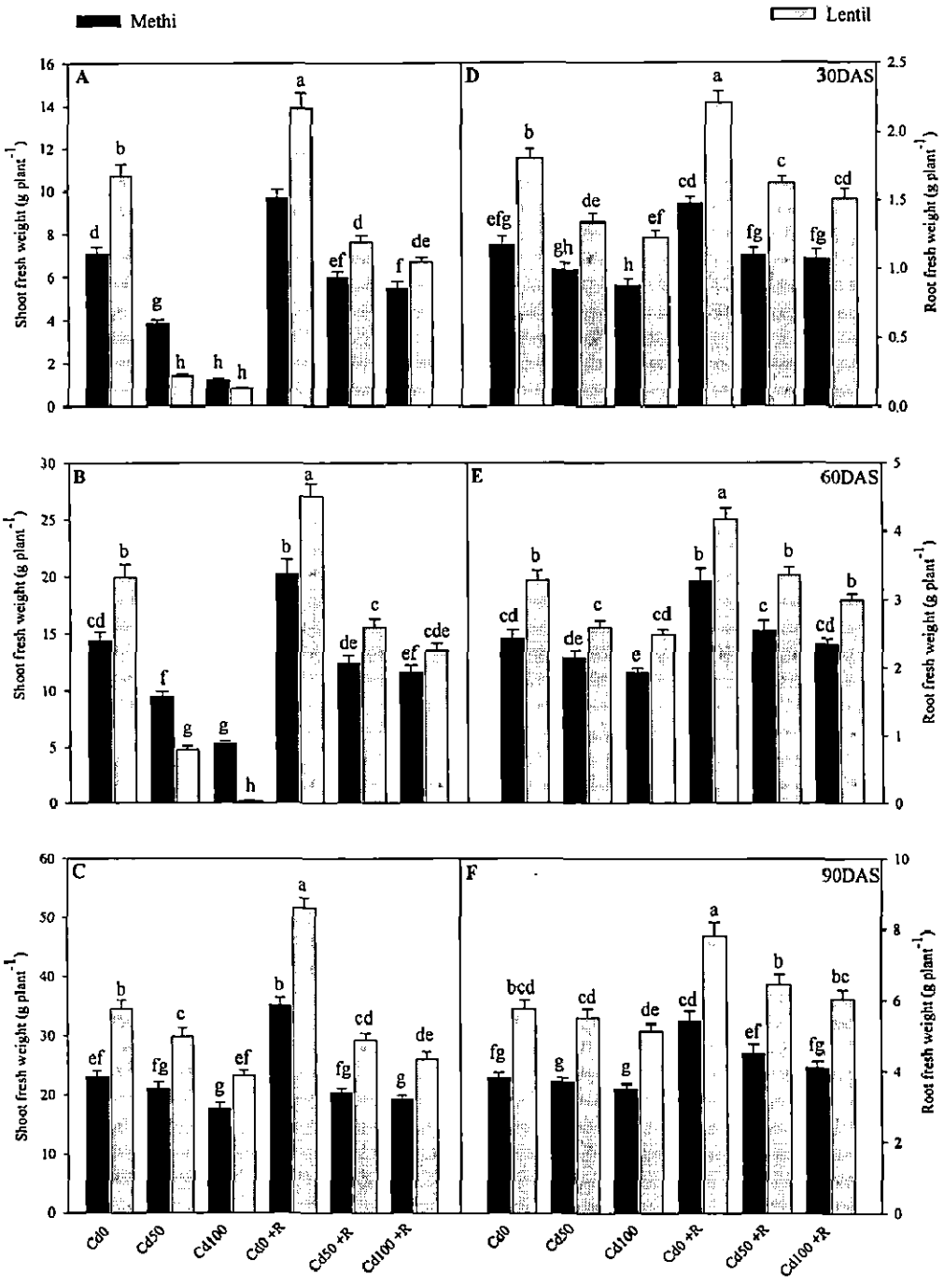


Figure 4.21: Effect of *Rhizobium* (R) inoculation against 0, 50 and 100 mg $CdCl_2$ Kg^{-1} soil on shoot fresh weight and root fresh weight (g $plant^{-1}$) of methi (*Trigonella foenum-graecum*) and lentil (*Lens culanaris*) at 30, 60 and 90 days after sowing.

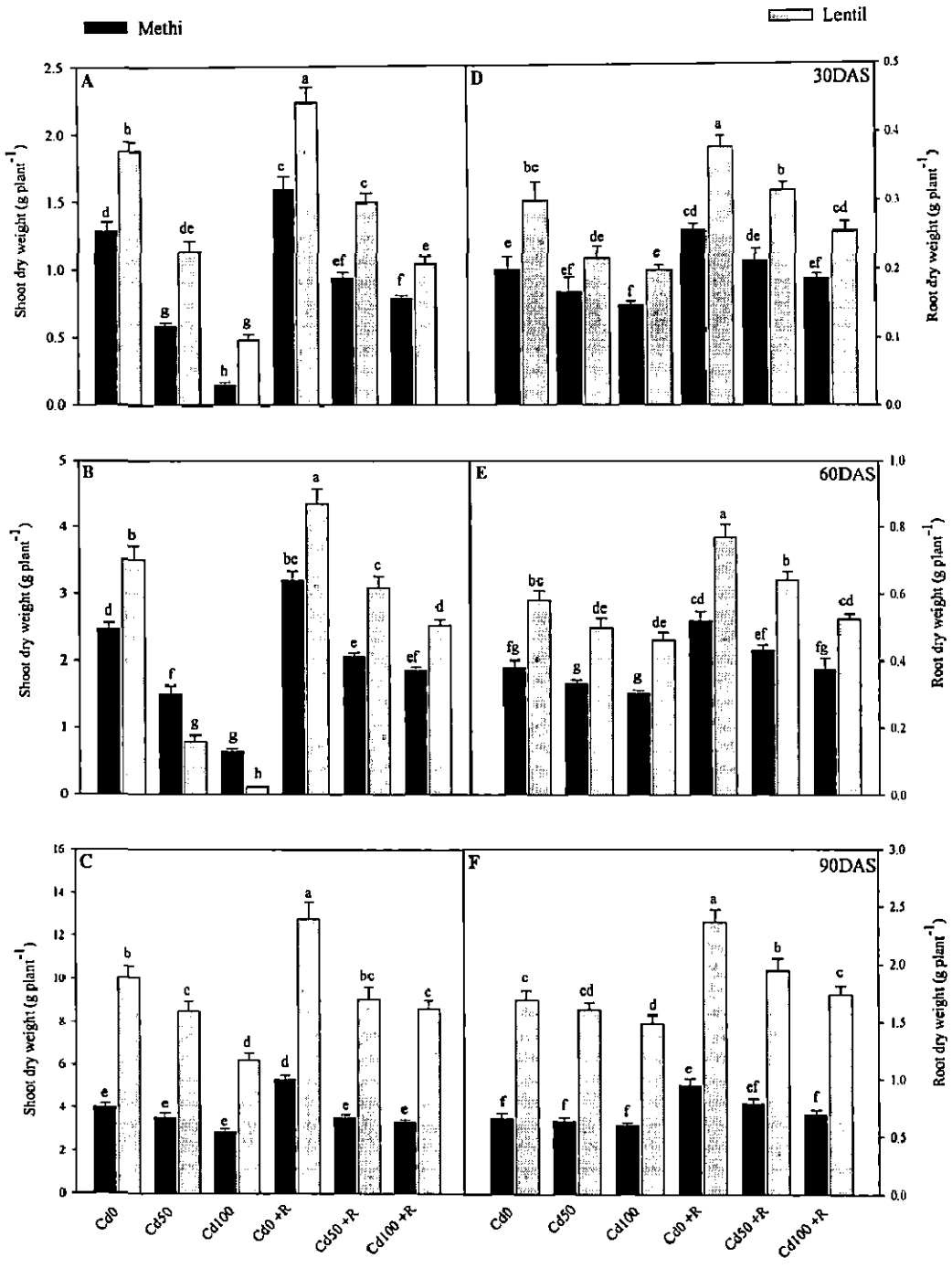


Figure 4.22: Effect of *Rhizobium* (R) inoculation against 0, 50 and 100 mg $CdCl_2$ Kg^{-1} soil on shoot dry weight and root dry weight (g $plant^{-1}$) of methi (*Trigonella foenum-graecum*) and lentil (*Lens culanaris*) at 30, 60 and 90 days after sowing.

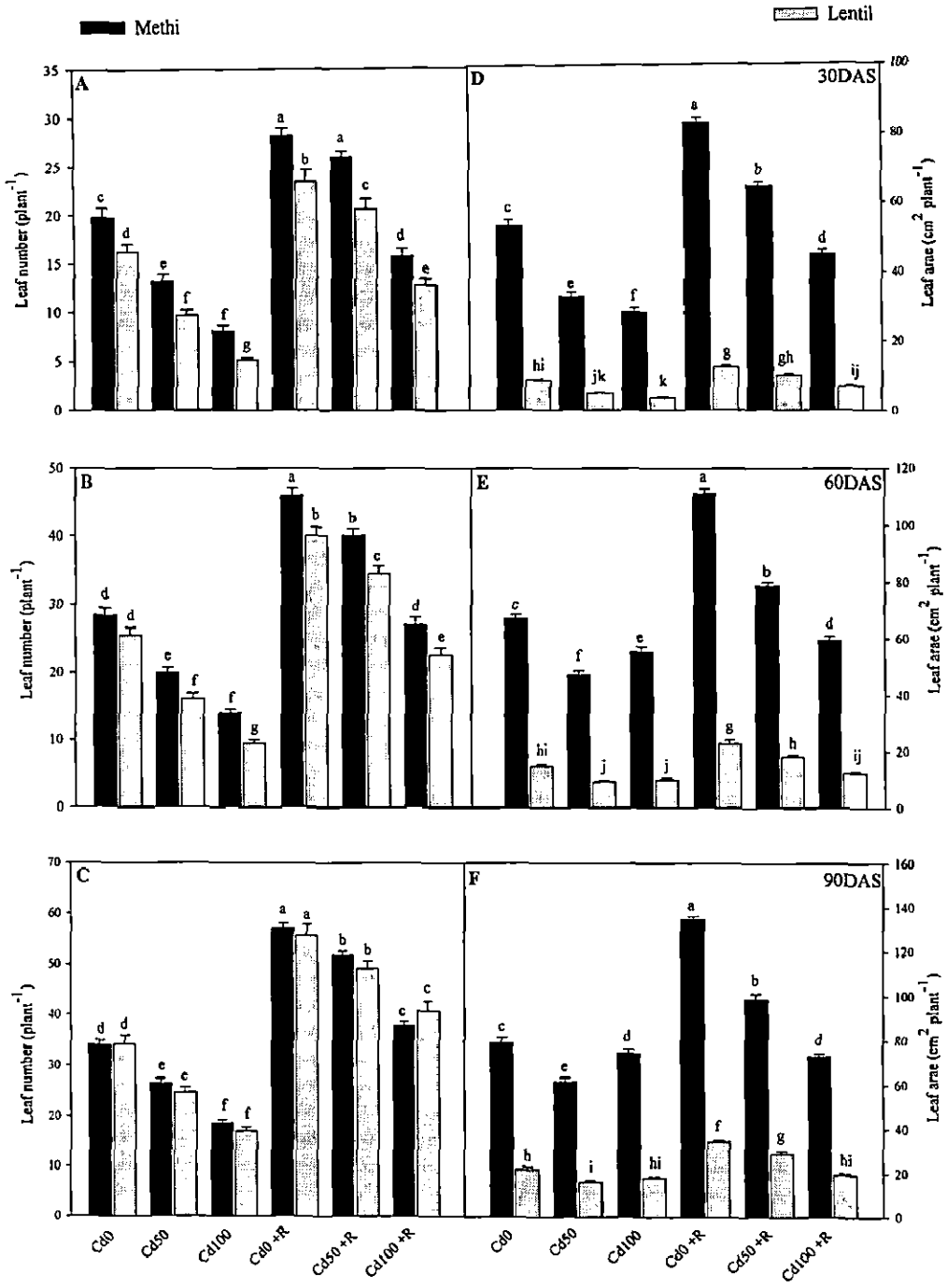


Figure 4.23: Effect of *Rhizobium* (R) inoculation against 0, 50 and 100 mg $CdCl_2$ Kg^{-1} soil on leaf number ($plant^{-1}$) and leaf area (cm^2 $plant^{-1}$) of methi (*Trigonella foenum-graecum*) and lentil (*Lens culanaris*) at 30, 60 and 90 days after sowing.

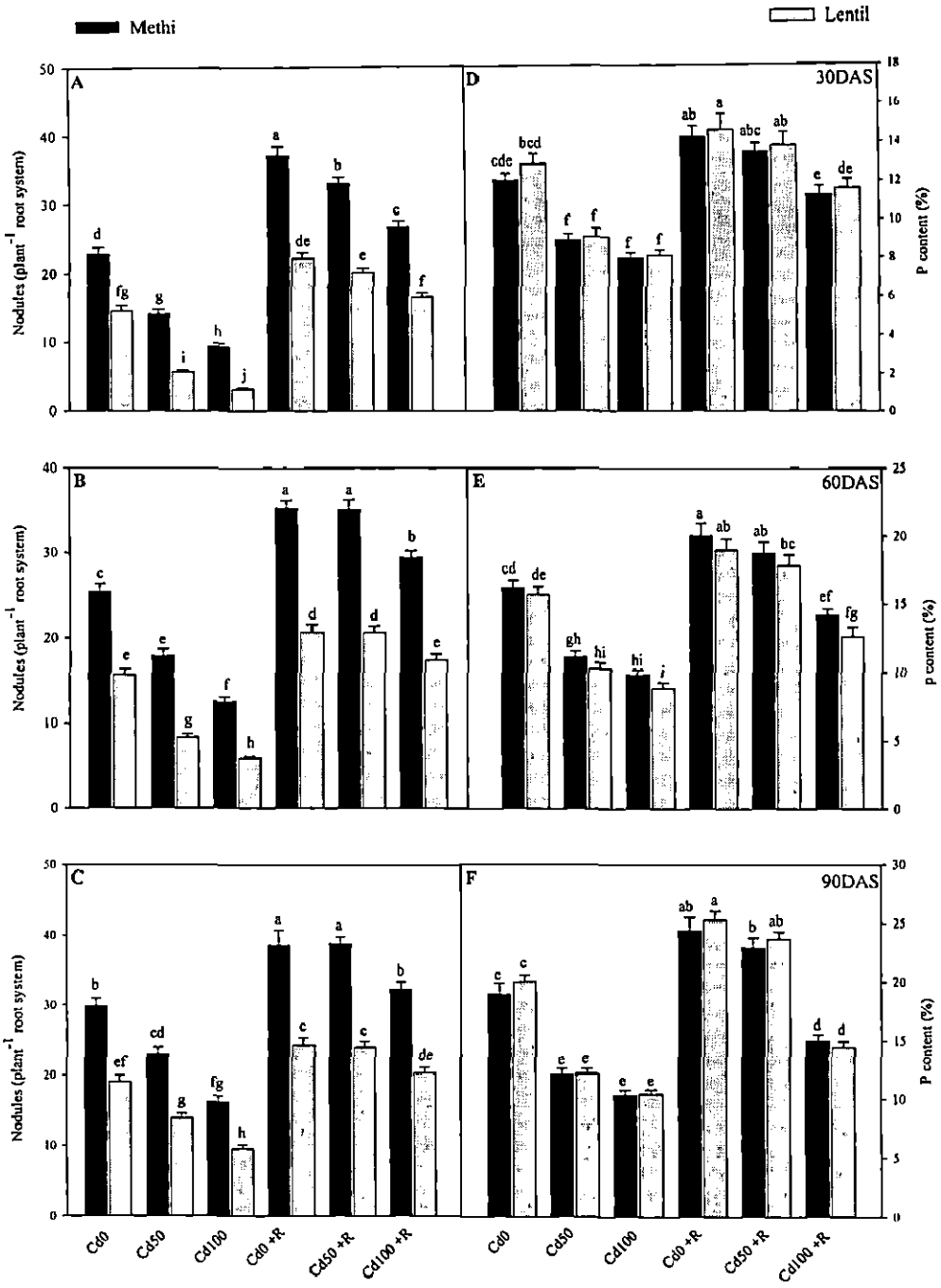


Figure 4.24: Effect of *Rhizobium* (R) inoculation against 0, 50 and 100 mg $CdCl_2$ Kg^{-1} soil on number of nodules ($root^{-1}$ system) and phosphorous content (%) of methi (*Trigonella foenum-graecum*) and lentil (*Lens culanaris*) at 30, 60 and 90 days after sowing.

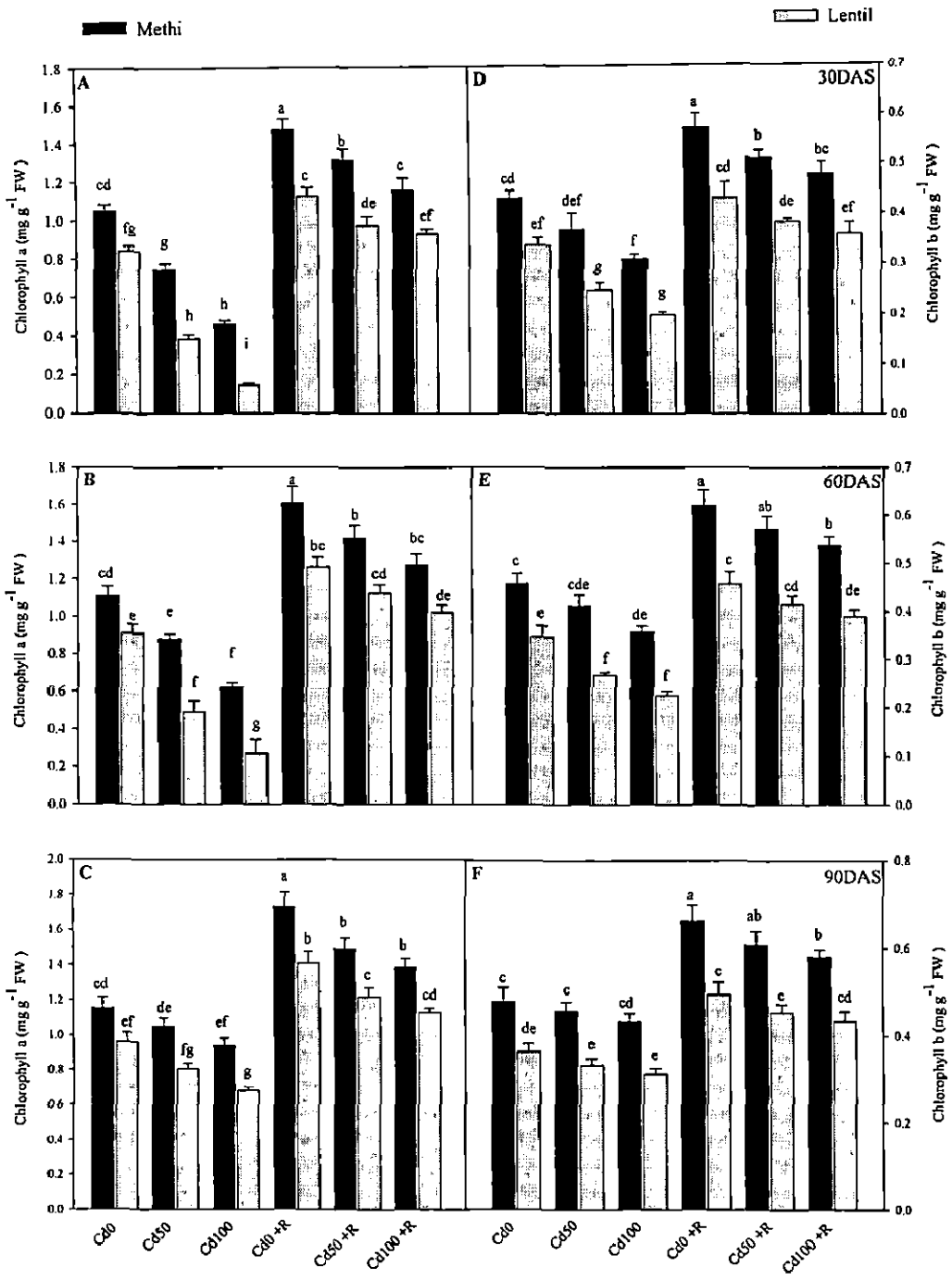


Figure 4.25: Effect of *Rhizobium* (R) inoculation against 0, 50 and 100 mg $CdCl_2$ Kg^{-1} soil on chlorophyll a (mg g^{-1} FW) and chlorophyll b (mg g^{-1} FW) of methi (*Trigonella foenum-graecum*) and lentil (*Lens culanaris*) at 30, 60 and 90 days after sowing.

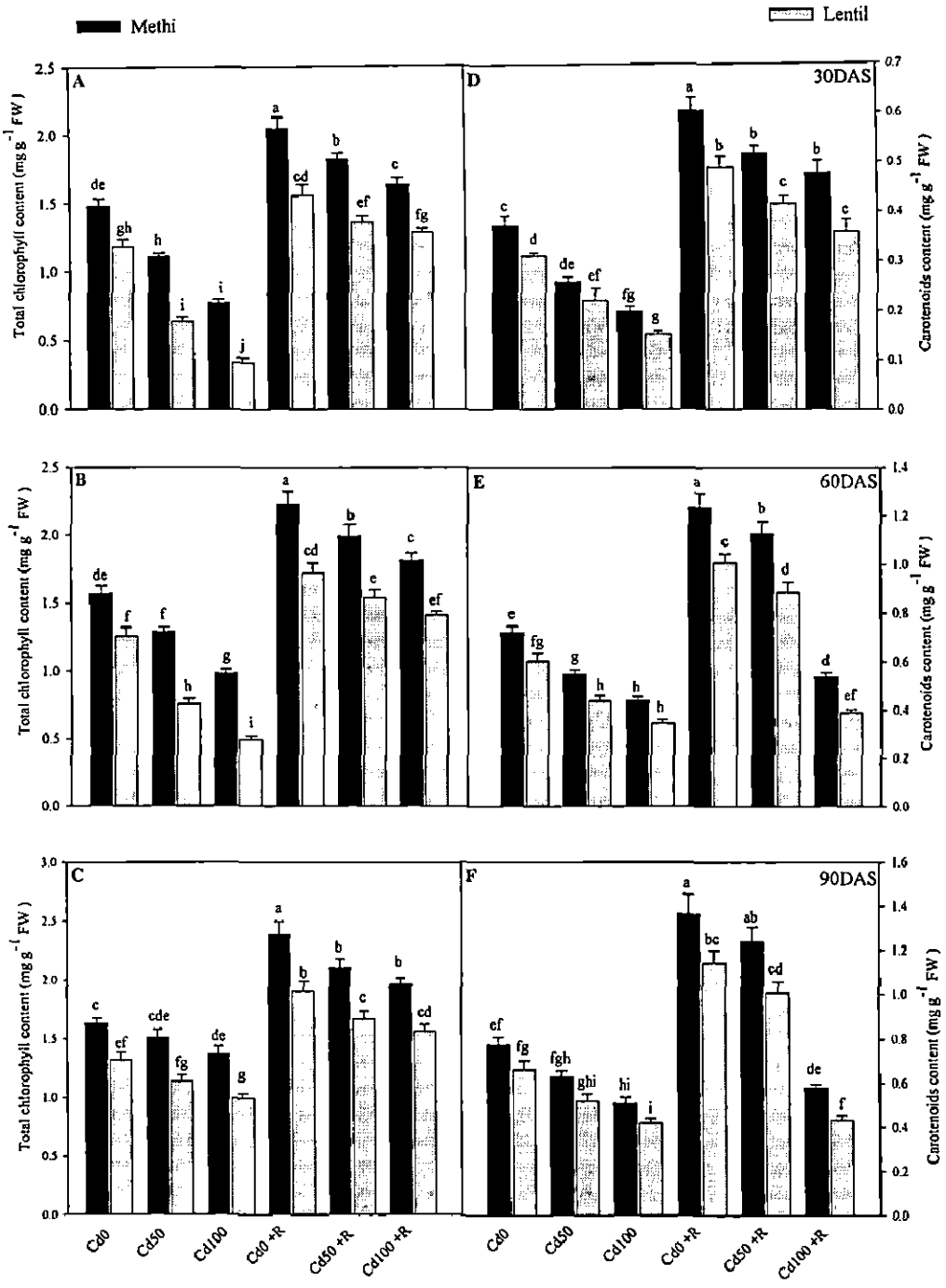


Figure 4.26: Effect of *Rhizobium* (R) inoculation against 0, 50 and 100 mg $CdCl_2$ Kg^{-1} soil on total chlorophyll (mg g^{-1} FW) and carotenoid contents (mg g^{-1} FW) of methi (*Trigonella foenum-graecum*) and lentil (*Lens culanaris*) at 30, 60 and 90 days after sowing.

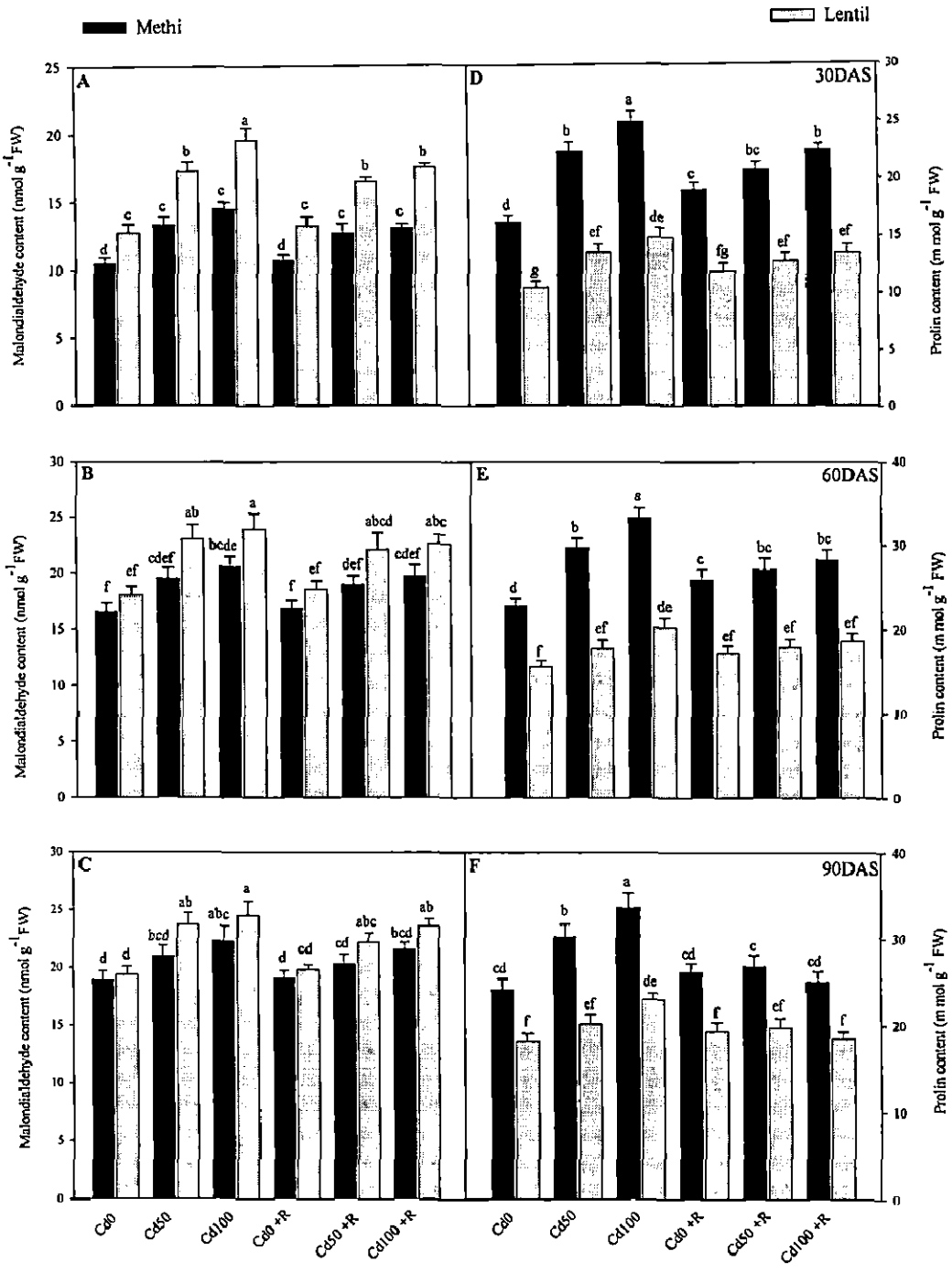


Figure 4.27: Effect of *Rhizobium* (R) inoculation against 0, 50 and 100 mg $CdCl_2$ Kg^{-1} soil on malondialdehyde (nmol $g^{-1}FW$) and proline content (mmol g^{-1} FW) of methi (*Trigonella foenum-graecum*) and lentil (*Lens culanaris*) at 30, 60 and 90 days after sowing.

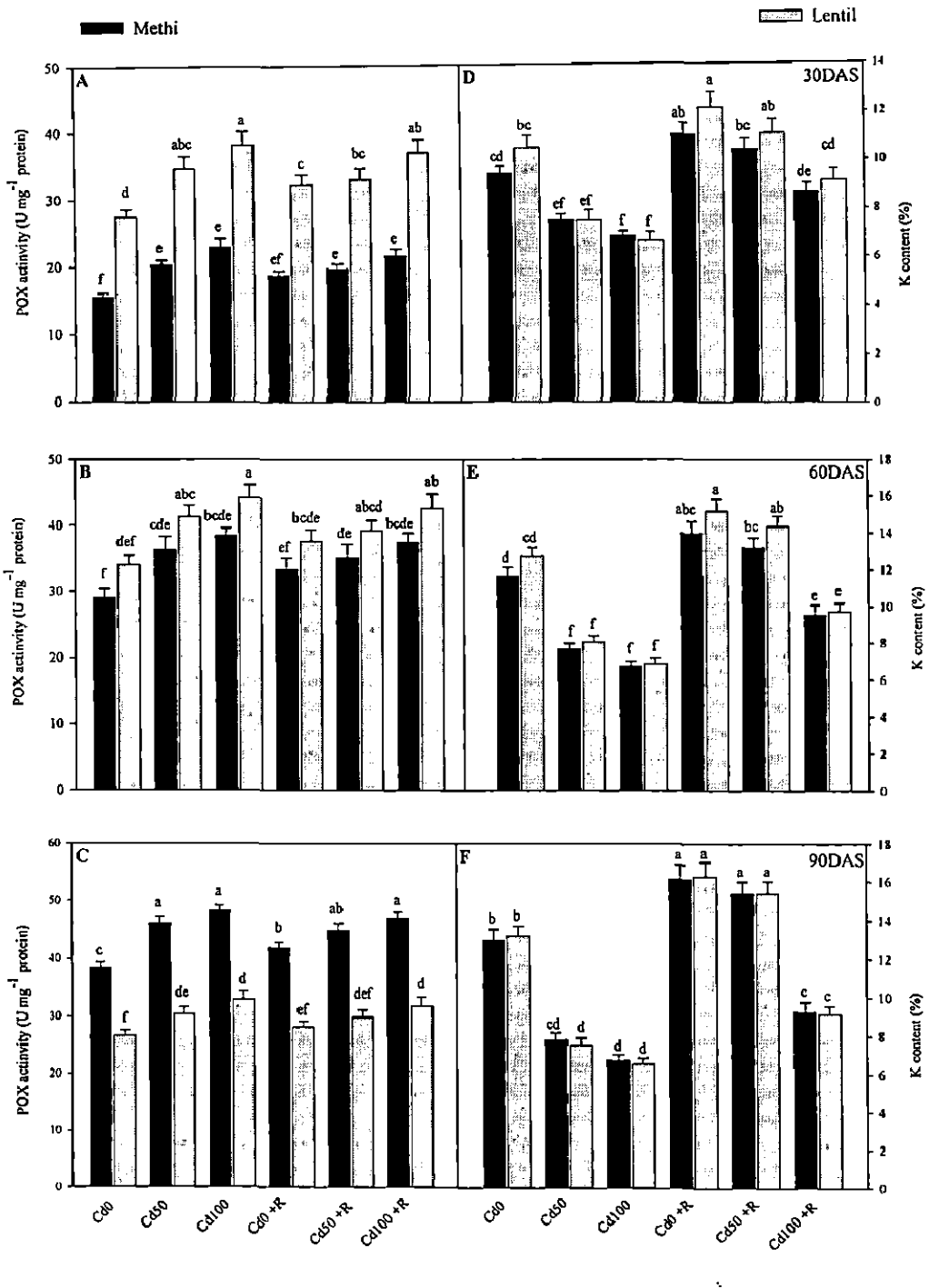


Figure 4.28: Effect of *Rhizobium* (R) inoculation against 0, 50 and 100 mg $CdCl_2$ Kg^{-1} soil on POX activity (U mg^{-1} protein) and potassium content (%) of methi (*Trigonella foenum-graecum*) and lentil (*Lens culanaris*) at 30, 60 and 90 days after sowing.

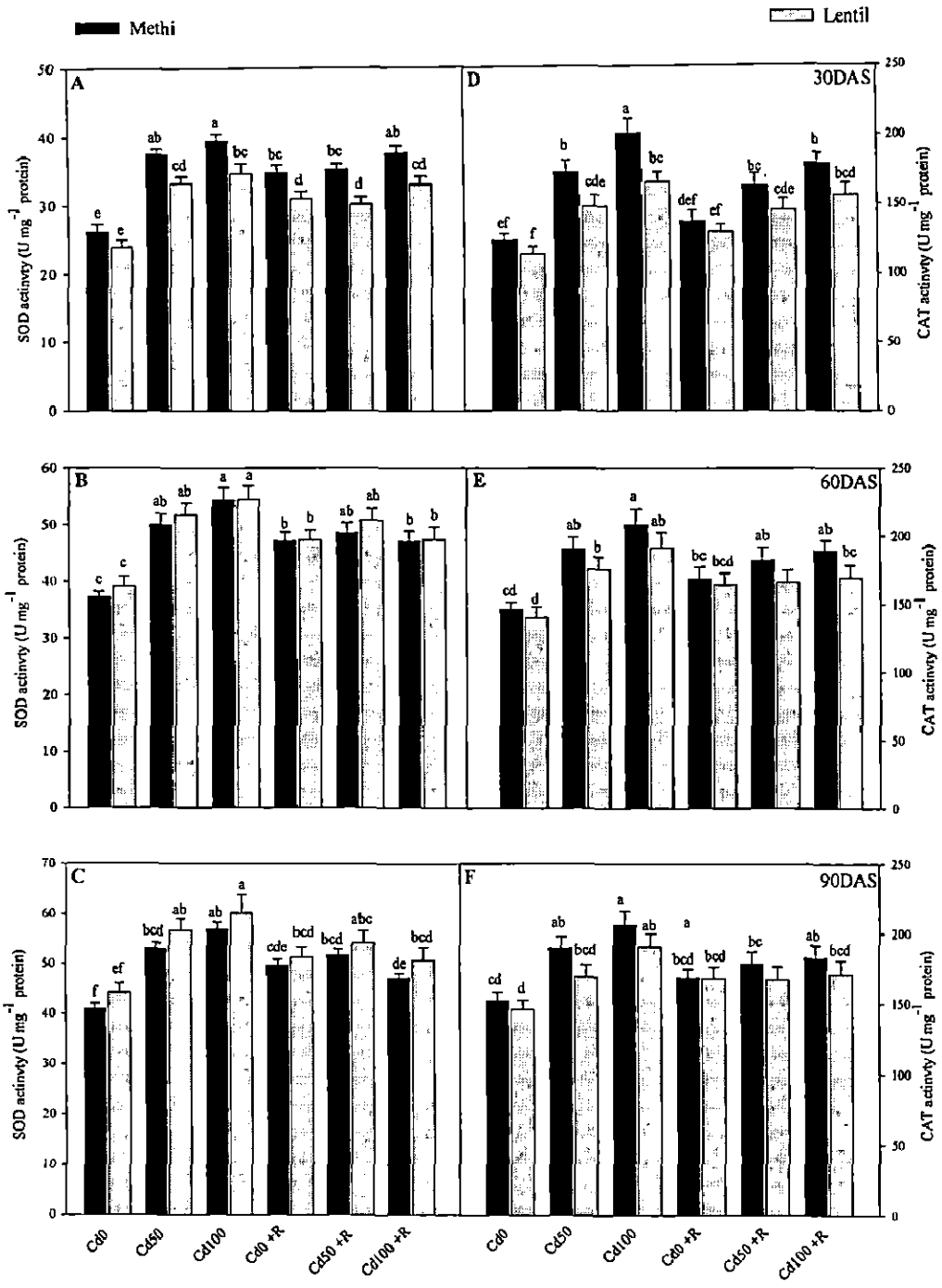


Figure 4.29: Effect of *Rhizobium* (R) inoculation against 0, 50 and 100 mg $CdCl_2$ Kg^{-1} soil on SOD activity (U mg^{-1}protein) and catalase activity (mM H_2O_2 decomposed g^{-1}FW) of methi (*Trigonella foenum-graecum*) and lentil (*Lens culanaris*) at 30, 60 and 90 days after sowing.

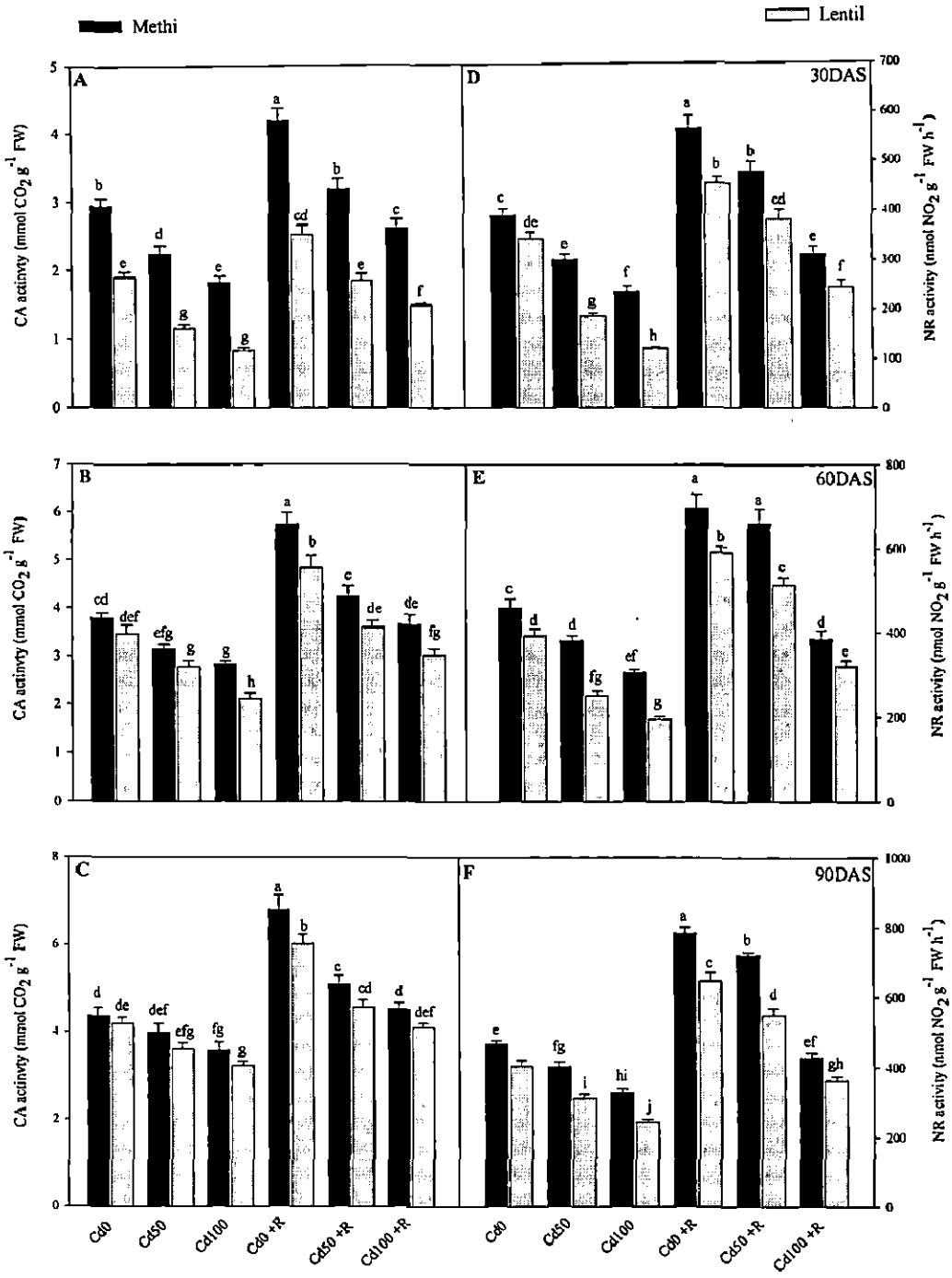


Figure 4.30: Effect of *Rhizobium* (R) inoculation against 0, 50 and 100 mg $CdCl_2$ Kg^{-1} soil on CA (mmol CO_2 g^{-1}FW) and NR activities (nmol NO_2 g^{-1} FW h^{-1}) of methi (*Trigonella foenum-graecum*) and lentil (*Lens culanaris*) at 30, 60 and 90 days after sowing.

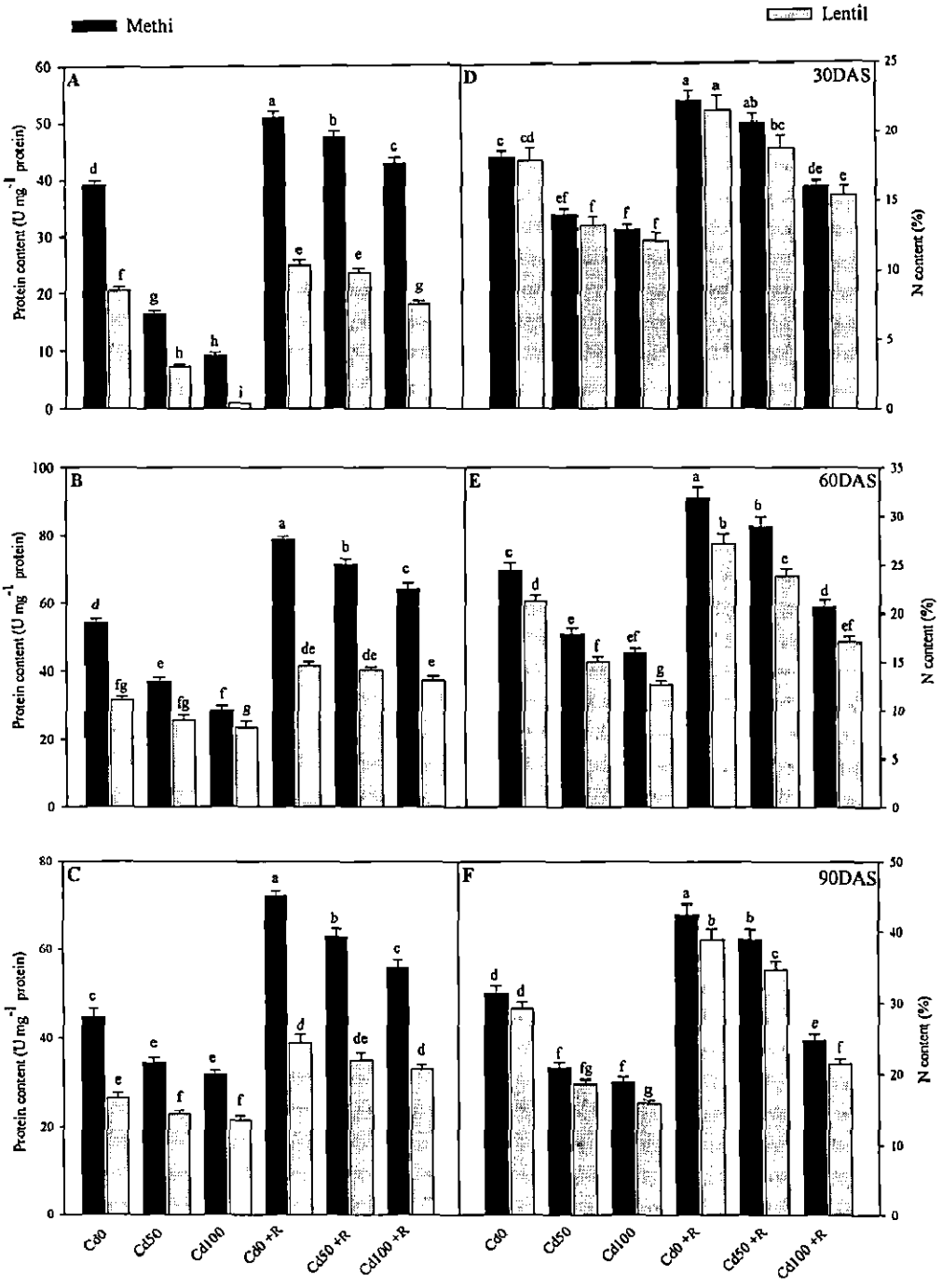


Figure 4.31: Effect of *Rhizobium* (R) inoculation against 0, 50 and 100 mg $CdCl_2$ Kg^{-1} soil on protein content (U mg^{-1} protein) and potassium (%) of methi (*Trigonella foenum-graecum*) and lentil (*Lens culanaris*) at 30, 60 and 90 days after sowing.

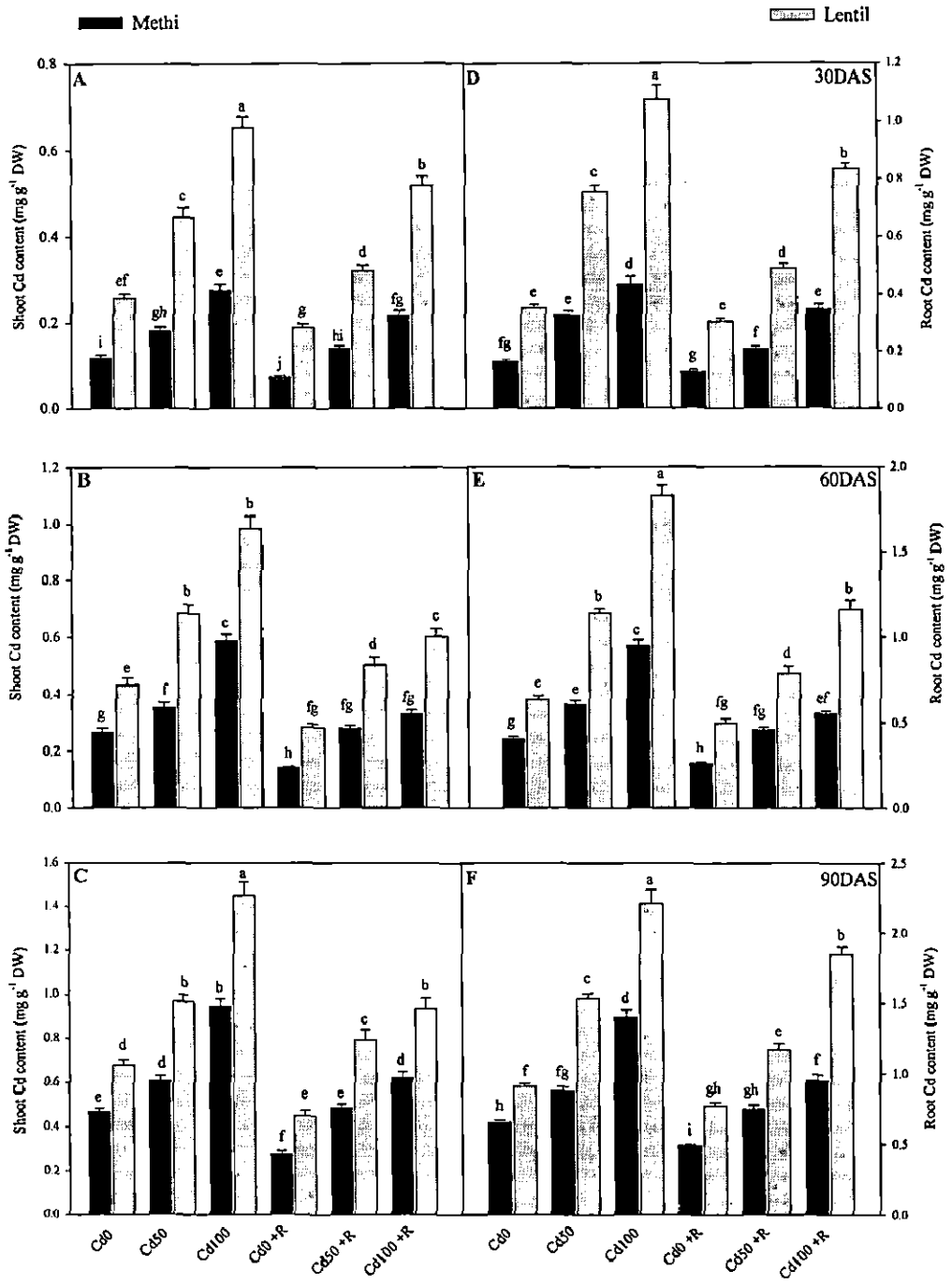


Figure 4.32: Effect of *Rhizobium* (R) inoculation against 0, 50 and 100 mg $CdCl_2$ Kg^{-1} soil on shoot Cd content (µg g^{-1} DW) and shoot Cd content (µg g^{-1} DW) of methi (*Trigonella foenum-graecum*) and lentil (*Lens culanaris*) at 30, 60 and 90 days after sowing.

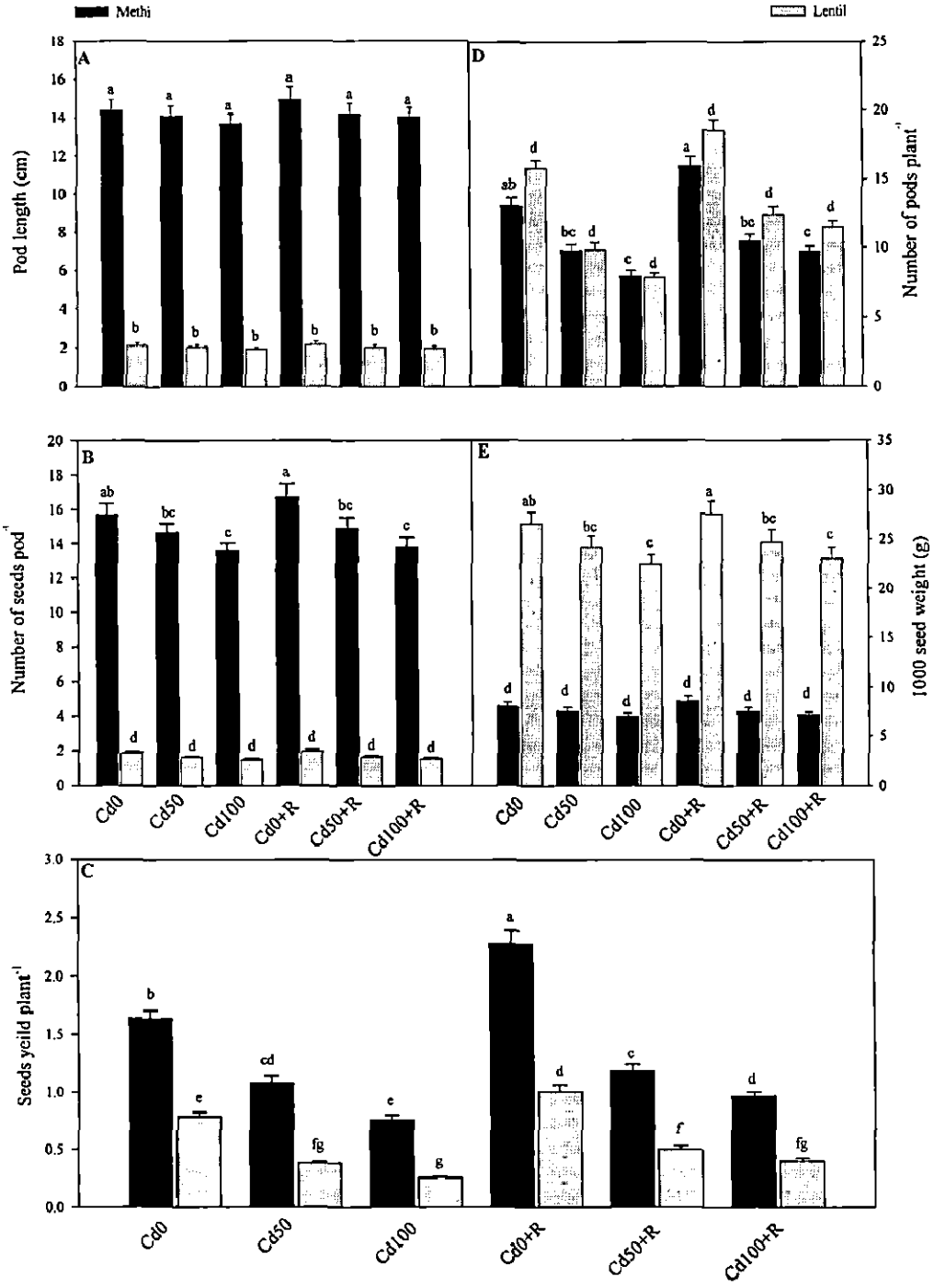


Figure 4.33: Effect of *Rhizobium* (R) inoculation against 0, 50 and 100 mg $CdCl_2$ Kg^{-1} on pod length (cm), number of pods $plant^{-1}$, number of seeds pod^{-1}, 1000 seeds weight and seed yield $plant^{-1}$ of methi (*Trigonella foenum-graecum*) and lentil (*Lens culanaris*) at harvest i.e., 120 days after sowing..

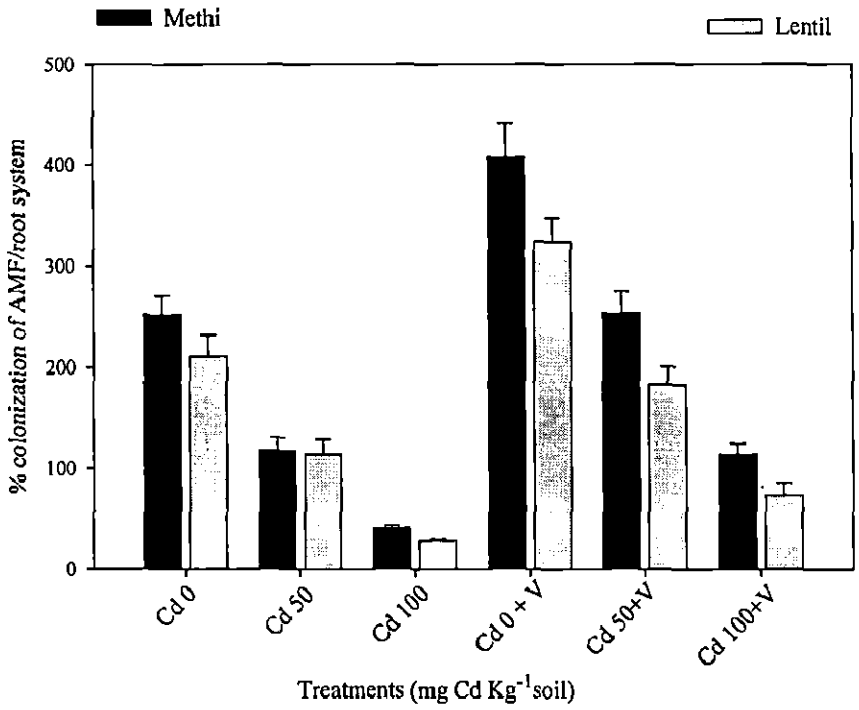


Figure 4.34: Tolerance index of five legumes (methi, broad bean, chick pea, pea, and lentil) exposed to 0, 50 and 100 mg Cd Kg^{-1} soil alone and with seed inoculated with *Rhizobium*. Tolerance index was calculated as per cent change of seed yields with control.

4.3 Experiment 3: To study the alleviation potential of AM fungi against Cd-induced effects in Cd sensitive and non-sensitive Cd legumes

Experiment 3 was conducted on the similar pattern as of Experiment 2 with an aim to study the effectiveness of AM fungi against Cd stress (50 mg and 100 mg Kg^{-1} soil) in methi (Cd non-sensitive) and lentil (Cd sensitive) legumes.

The details of results briefly described below and summarized in Figures (4.35 – 4.50)

4.3.1 Growth characteristics

Application of AM fungi alleviated the negative effects on growth parameters (root and shoot length, fresh and dry weights, leaf numbers and area per plant and number of nodules per root system) of methi and lentil due to Cd amendment (Cd 50 or 100 mg Kg^{-1}) in soil (Figures 4.35 - 4.39). The application of AM fungi completely nullified the adverse effects of 50 mg Cd Kg^{-1} soil for the observed growth parameters. However, AM fungi also mitigated the adverse effect of 100 mg Cd Kg^{-1} soil on growth parameters at all growth stages. Arbuscular mycorrhizal inoculation increased all the growth characteristics. However, this increase was more in methi than lentil. The growth improved with the age of the plant (30 to 90 DAS). Shoot and root length, root fresh and dry weights, leaf number per plant, leaf area per plant and number of nodules per root system in methi was decreased by 37.8, 19.3, 15.2, 16.4, 30.2, 38.4 and 38.1% respectively, compared to control due to Cd 50 mg Cd Kg^{-1} soil at 30 DAS. Contrarily, AM inoculation increased shoot and root length, root fresh and dry weight, leaf number per plant, leaf area per plant and number of nodules per root system by 20.3, 26.4, 10.2, 9.9, 36.1, 27.3 and 52.4% respectively, over the control in soil amended with 50 mg Cd Kg^{-1} soil at 30 DAS. However, AM inoculation increased shoot fresh and dry weight but this increase was at par with the control plants. AM inoculation gave a substantial gain of 3.5, 7.9% in shoot fresh weight and 5.0, 1.2% in shoot dry weight in methi and lentil respectively, compared to control, soil amended with 100 mg Cd Kg^{-1} soil at 30 DAS.

4.3.2 Photosynthetic attributes

Cadmium amendment in soil (50 or 100 mg Kg^{-1} soil) decreased the content of photosynthetic pigments (Figures 4.40 - 4.41) (chlorophyll a, chlorophyll b, total chlorophyll content and carotenoids content) at all the growth stages (Figures 4.40 -

4.41). The effect of Cd on these pigments was in order chlorophyll b < total chlorophyll < carotenoid content < chlorophyll a. The effect of Cd was more pronounced in lentil as compared to methi. Chlorophyll a and carotenoids content was decreased by 28.8, 30.8% in methi and 53.8, 34.7% in lentil respectively, over the control at 30 DAS due to 50 Cd mg Kg^{-1} of soil. Arbuscular mycorrhizal inoculation completely nullified the effects of 50 and 100 mg Cd Kg^{-1} soils at all growth stages and also in both the legumes AM inoculation increased the chlorophyll a and carotenoids content by 27.6% in methi and 40.8% in lentil respectively, compared to control at 30 DAS. Inoculation of AM fungi maximally increased the pigment contents in all the treatments.

4.3.3 Metabolic stress markers: Lipid peroxidation and proline content

The content of MDA and proline in the leaves of the two legumes increased with age and also with the increase in the level of Cd in soil (Figure 4.42). Application of 50 mg Cd Kg^{-1} soil raised the MDA and proline content of plants, more at early growth stages (30 or 60 DAS) as compared to later growth stage (90 DAS) 100 mg Cd Kg^{-1} soil gave maximum increase in MDA and proline content by 38.1, 54.3% in methi and 38.1, 41.8% in lentil respectively, over the control at 30 DAS. Inoculation of AM fungi decreased these parameters close to control plants but was significantly higher. Arbuscular mycorrhizal inoculated plants supplemented with 100 mg Cd Kg^{-1} soil was at par to that of un-inoculated plants treated with 50 mg Cd Kg^{-1} soil at 30 DAS. Out of the two legumes, the contents of these metabolites were higher in lentil as compared methi.

4.3.4 Enzymatic stress markers: Antioxidant activity

The activity of antioxidant enzymes increased with the increase in the level of Cd in soil and also the activity was more in lentil than methi (Figures 4.43 - 4.44). In methi, 100 mg Cd Kg^{-1} soil increased the activity of POX, CAT and SOD by 42.43, 40.17 and 45.26% respectively, over the control at 30 DAS. However, Cd amendment increased the activity of POX, CAT and SOD by 50.20, 45.28, and 54.07% respectively, as compared to control plants at 30 DAS. AM inoculation significantly increased the enzyme activities in both the legumes and this increase was more in methi but their activity decreased with the age of plants. In AM treated plants, CAT

and SOD activity was increased by 40.2 and 45.3% in methi by 31.0, 39.2% in lentil respectively, as compared to control.

4.3.5 Carbonic anhydrase and nitrate reductase activities

Carbonic anhydrase and NR activity along with total protein content decreased with the increase in the level of Cd in soil (Figures 4.45). NR and CA activity was increased by 22.4, 23.6% in methi and 45.7, 39.8% in lentil respectively, over the control at 30 DAS. However, in plants treated with Cd (50 or 100 mg Kg^{-1} soil) protein content increased up to 30 DAS thereafter, it declined. Inoculation of AM fungi further enhanced CA and NR activity along with total protein content in both the legumes however, methi showed maximum significant increase in these attributes compared to lentil, irrespective of the treatments. AM fungi partially reduced the toxicity of 100 mg Cd Kg^{-1} soil.

4.3.6 Leaf protein content

The leaf protein content also followed the similar trend for treatments as that of the enzymes. However, it was comparatively higher in AM inoculated plants compared to non-inoculated plants supplemented with 50 or 100 mg Cd Kg^{-1} soil (Figure 4.46).The per cent increase in protein content of leaf was highest at 60 DAS as compared to early or later growth stages. Addition of Cd decreased the protein content. However, this decrease was more with 100 mg Cd Kg^{-1} soil. A decline of 60.0 and 64.0% in methi and lentil respectively, as compared to control due to 50 mg Cd Kg^{-1} soil at 30 DAS. Plants inoculated with *Rhizobium* showed increase of 34.0 and 18.3% in both the legumes due to 50 mg Cd Kg^{-1} soil at 60 DAS as compared to control.

4.3.7 N, P and K content in leaves

Leaf N, P and K contents decreased significantly with the increase in the level of Cd in soil (Figures 4.46 - 4.43). AM application in soil significantly increased N, P and K contents in both the plants supplemented with 50 mg Cd Kg^{-1} soil. However, its application increased the N and P contents insignificantly in the plants treated with Cd 100 mg Kg^{-1} soil as compared to control plants. The decrease of N, P and K contents in methi was 35.6, 44.0 and 47.8% due to 50 mg Cd Kg^{-1} was at 90 DAS whereas, when co-inoculated with AM fungi it was increased 48.3, 29.1, 25.3%, compared to control. Methi retained higher contents of N, P and K in its leaves than lentil in

treatment independent manner. Furthermore, inoculation of AM fungi recovered the Cd- mediated decline of these minerals and significantly increased the N, P and K content in the leaves. The inoculation of AM fungi recorded maximum level of N, P and K content in the leaves in control.

4.3.8 Cadmium accumulation in root and shoot

In both the legumes, Cd accumulation increased as the growth progressed from 30 to 90 DAS (Figure 4.47). Amendment of Cd to soil further enhanced its content in the root and shoot and this enhancement increased with the increase in the level of Cd in soil. Root accumulated more Cd than shoot. Out of the two legumes, lentil showed more accumulation than methi. Lentil showed an accumulation of 1.6, 2.2 μg Cd g^{-1} dry weight in root whereas in shoot it was 1.0, 1.5 μg g^{-1} dry weight, due to 50 and 100 mg Cd Kg^{-1} soil at 90 DAS. AM fungi inoculations with seeds significantly decreased the Cd accumulation.

4.3.9 Yield characteristics

Yield characteristics (pod length, number of pods per plant, number of seeds per pod, 1000 seed weight and seed yield per plant) decreased with the increase in the level of Cd in soil (Figure 4.48). The per cent decrease of all the yield attributes was more in lentil as compared to methi. A decrease of 47.0, 50.6% in number of pods per plant; 52.7, 56.3% in seed yield per plant was observed in methi and lentil respectively, over the control due to 100 mg Cd Kg^{-1} soil. Application of AM fungi partially recovered this decline for all the yield parameters. However, recovery was more in the plants treated with 50 mg Cd Kg^{-1} soil. AM inoculation increased the number of pods per plant by 2.0 and 2.7% and seed yield by 5.4 and 13.2% in methi and lentil respectively, supplemented with 50 mg Cd Kg^{-1} soil over the control. However, AM inoculation in control plants caused maximum increase in yield attributes.

4.3.10 Tolerance index

The tolerance index of legumes was calculated in terms of decrease in yield with the increasing doses of Cd (Figure 4.49). Methi showed highest tolerance followed by broad bean, chick pea and pea whereas lentil emerged as the least tolerant. Inoculation of AM fungi increased the tolerance of both the plants but this increase was more in methi than lentil. The inoculation showed an increase of 5.4, 33.0% in methi and 13.2, 14.2% in lentil due to 50 or 100 mg Cd Kg^{-1} soil respectively.

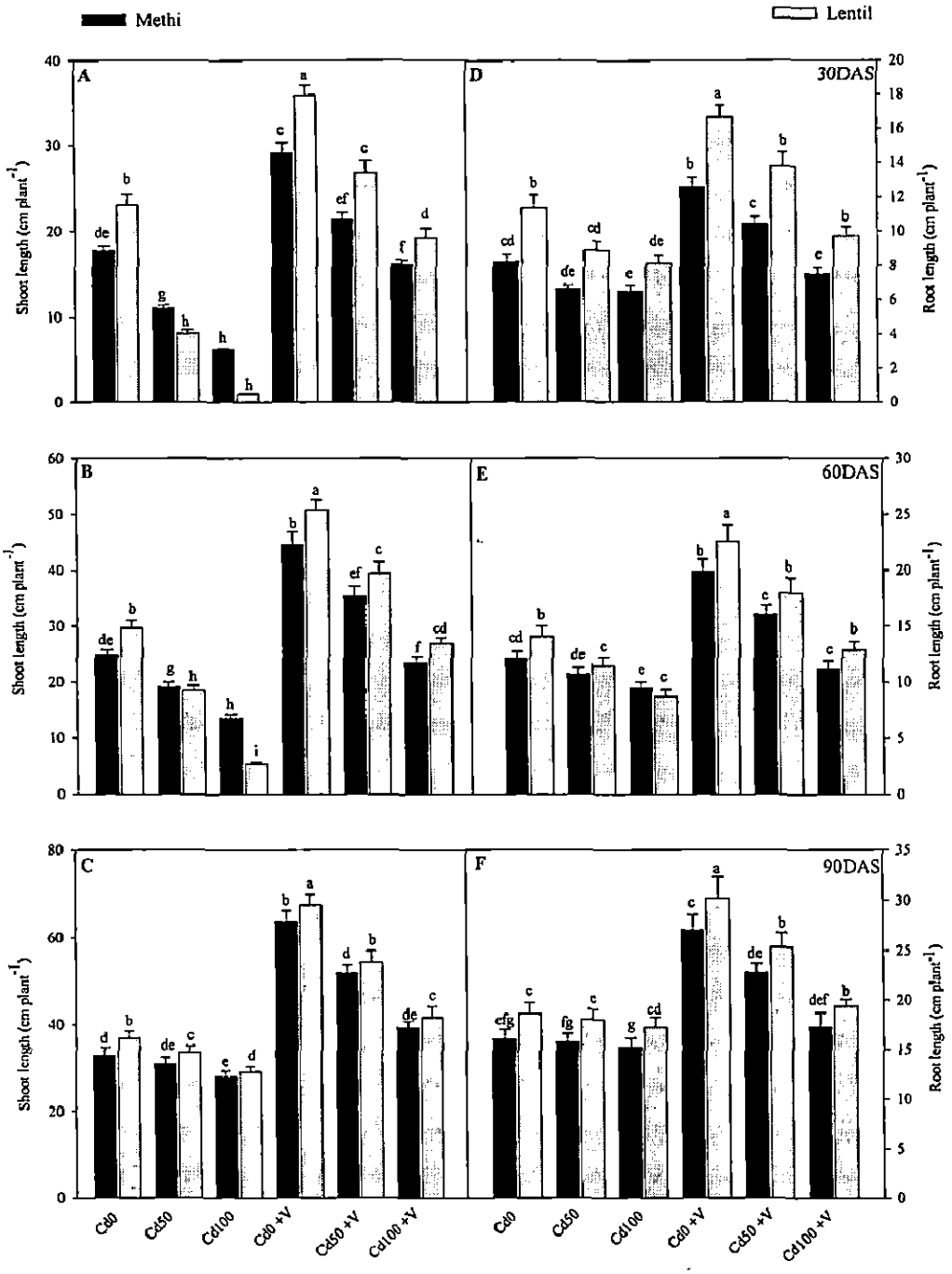


Figure 4.35: Effect of Arbuscular Mycorhizal (AM) fungi application against 0, 50 and 100 mg $CdCl_2$ Kg^{-1} soil on shoot length (cm) and root length of methi (*Trigonella foenum-graecum*) and lentil (*Lens culanaris*) at 30, 60 and 90 days after sowing.

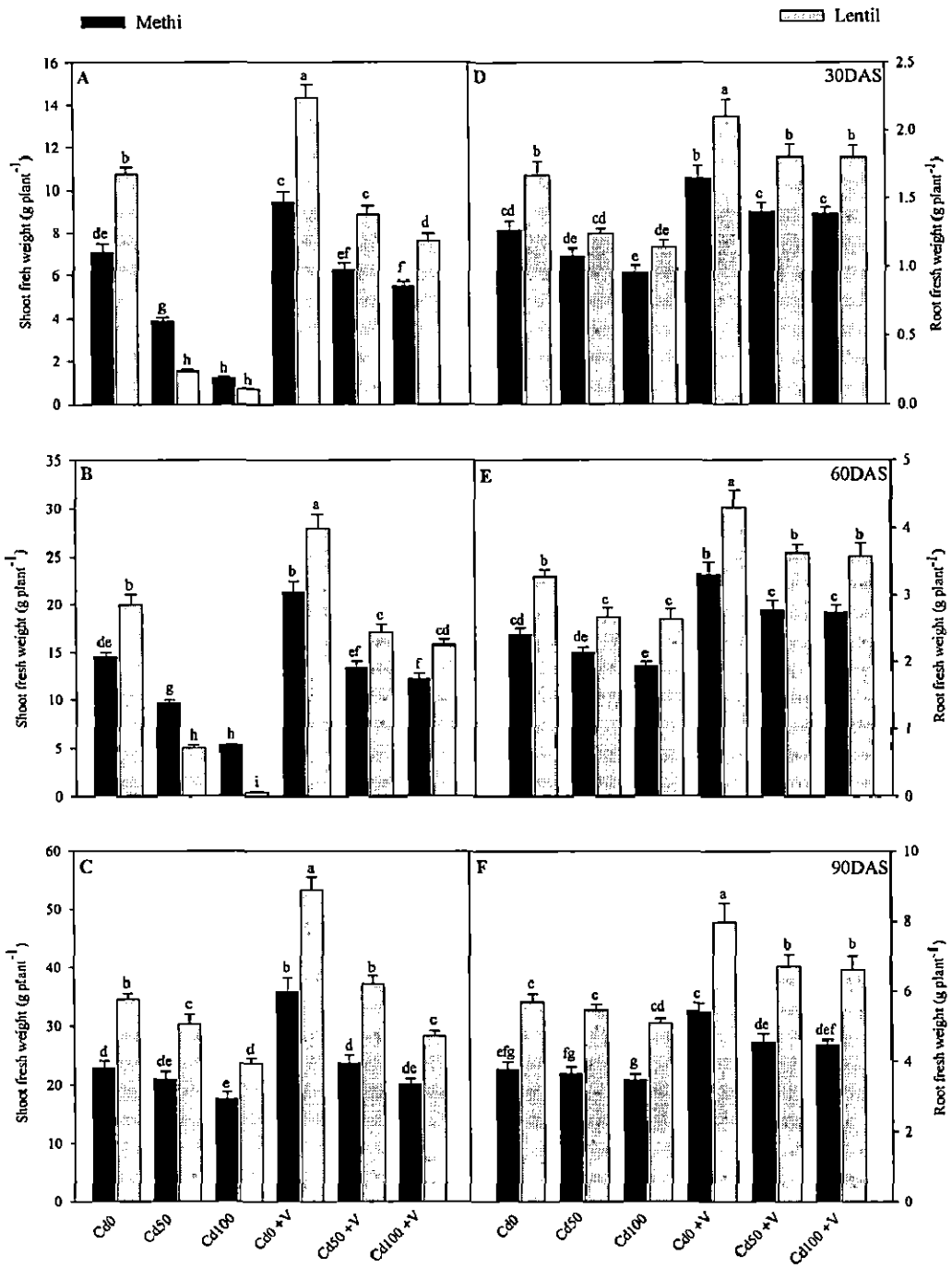


Figure 4.36: Effect of Arbuscular Mycorhizal (AM) fungi application against 0, 50 and 100 mg $CdCl_2$ Kg^{-1} soil on shoot fresh weight and root fresh weight (g $plant^{-1}$) of methi (*Trigonella foenum-graecum*) and lentil (*Lens culanaris*) at 30, 60 and 90 days after sowing.

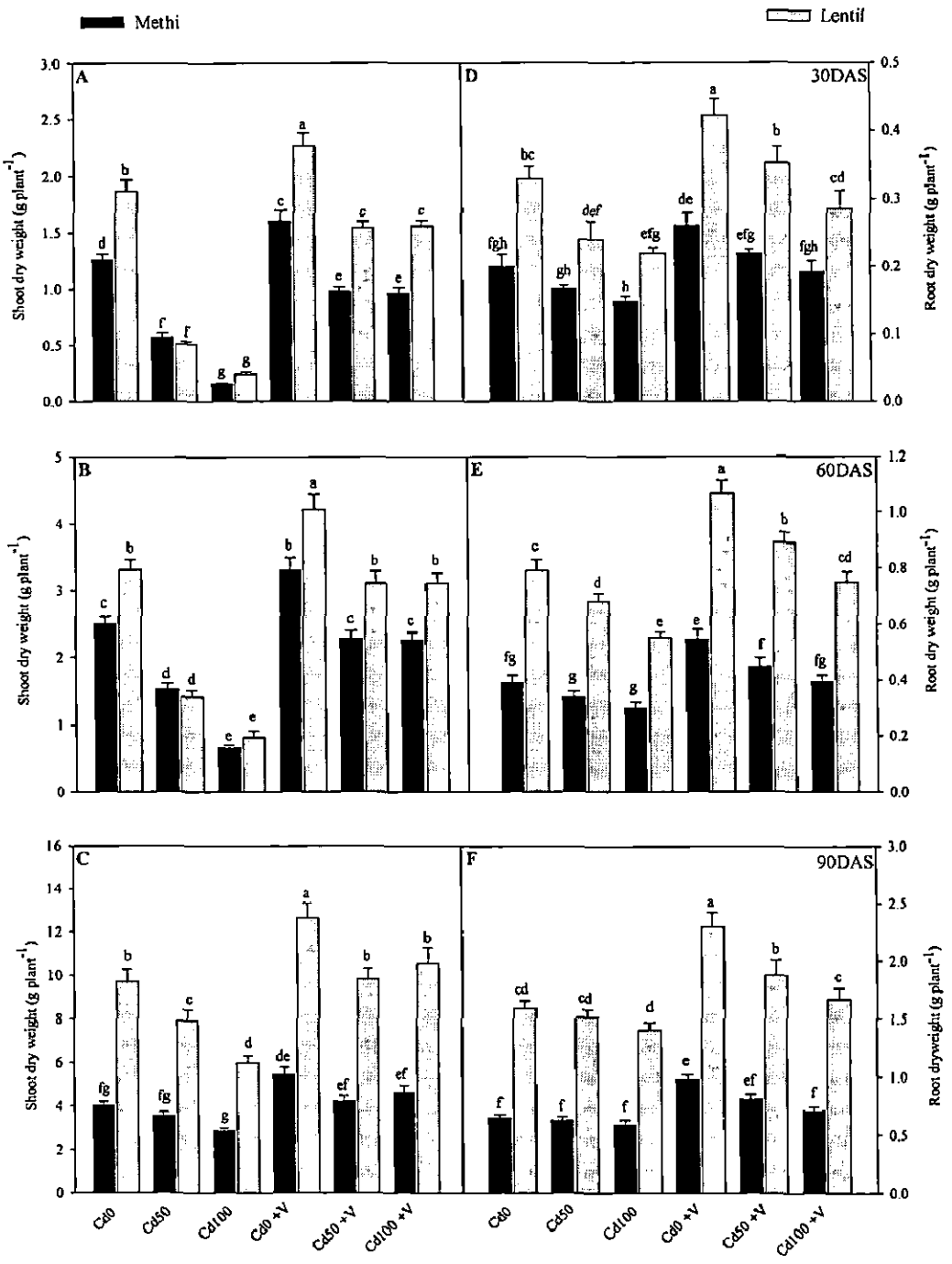


Figure 4.37: Effect of Arbuscular Mycorhizal (AM) fungi application against 0, 50 and 100 mg $CdCl_2$ Kg^{-1} soil on shoot dry weight and root dry weight (g $plant^{-1}$) of methi (*Trigonella foenum-graecum*) and lentil (*Lens culanaris*) at 30, 60 and 90 days after sowing.

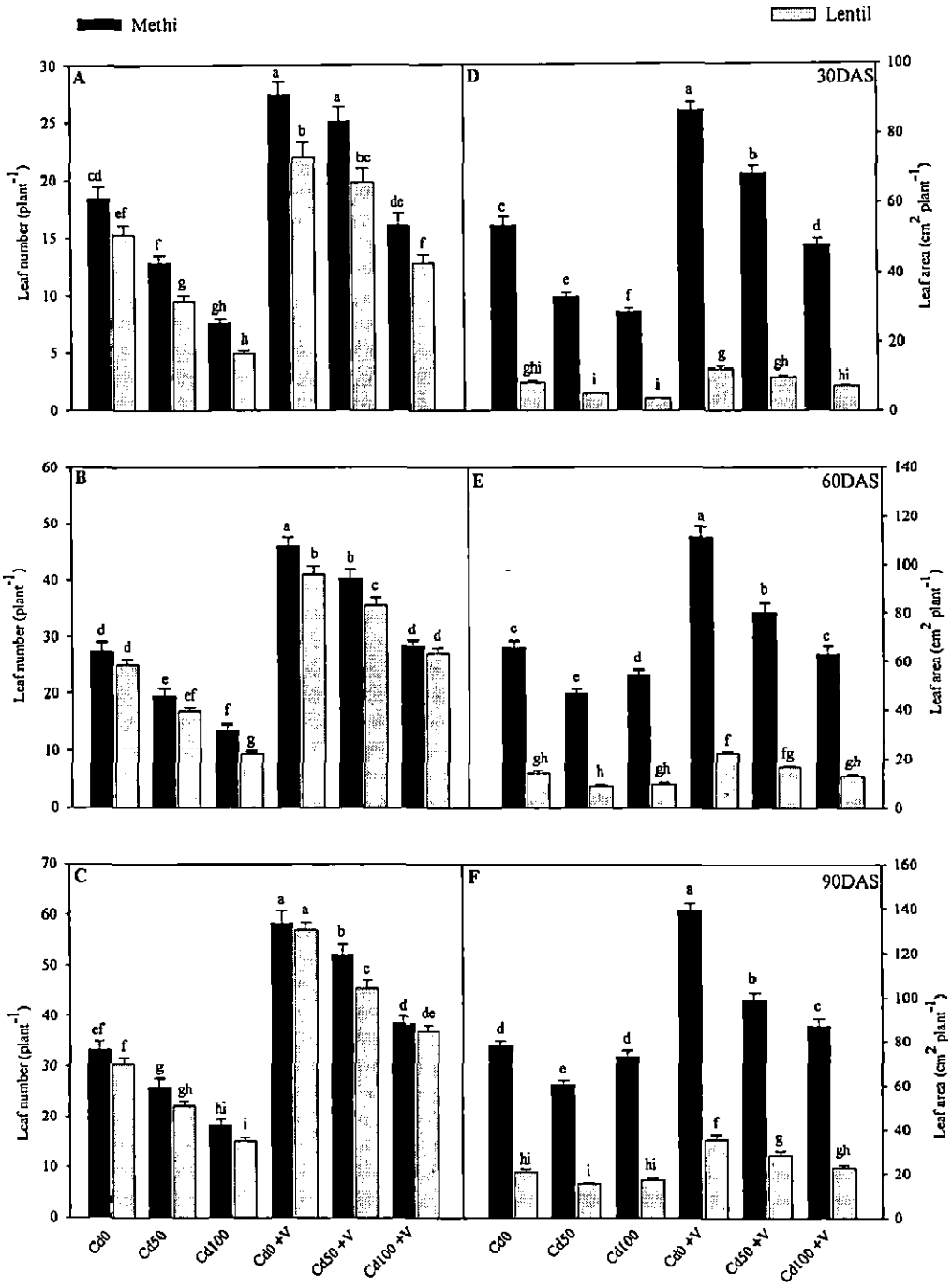


Figure 4.38: Effect of Arbuscular Mycorhizal (AM) fungi application against 0, 50 and 100 mg $CdCl_2$ Kg^{-1} soil on leaf number and leaf area (cm^2) of methi (*Trigonella foenum-graecum*) and lentil (*Lens culanaris*) at 30, 60 and 90 days after sowing.

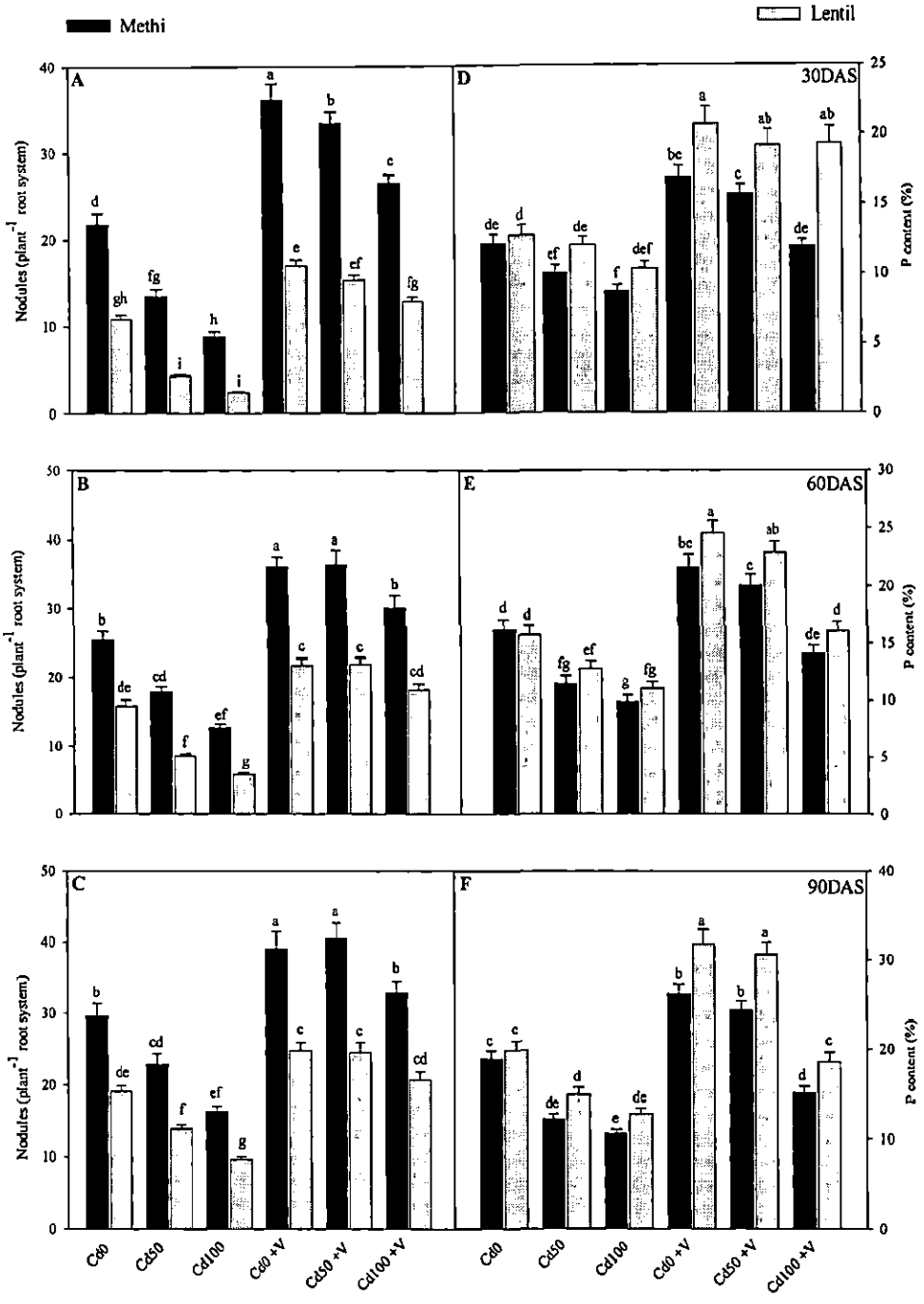


Figure 4.39: Effect of Arbuscular Mycorhizal (AM) fungi application against 0, 50 and 100 mg $CdCl_2$ Kg^{-1} soil on number of nodules (root^{-1} system) and phosphorous content (%) of methi (*Trigonella foenum-graecum*) and lentil (*Lens culanaris*) at 30, 60 and 90 days after sowing.

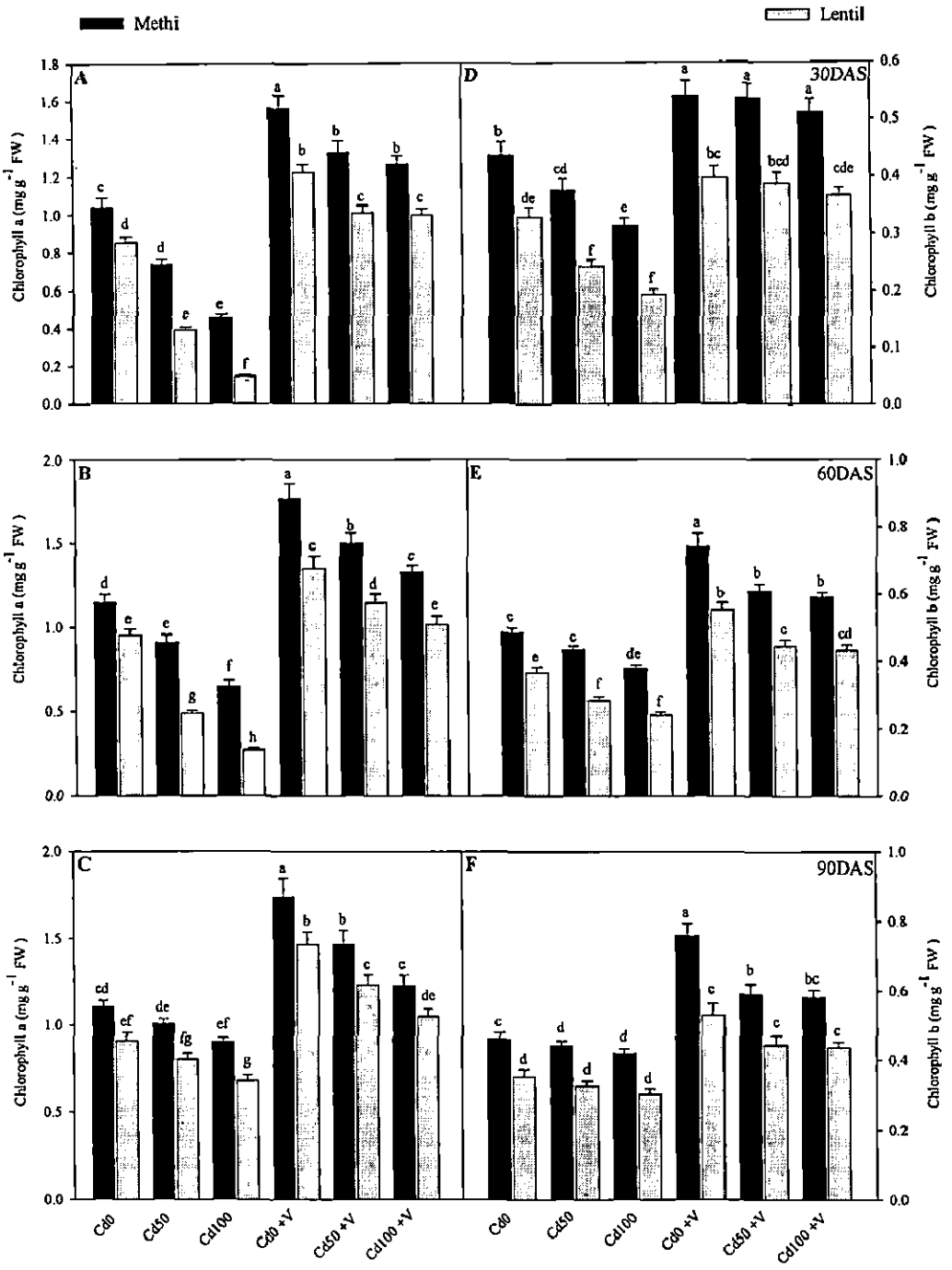


Figure 4.40: Effect of Arbuscular Mycorhizal (AM) fungi application against 0, 50 and 100 mg $CdCl_2$ Kg^{-1} soil on chlorophyll a (mg g^{-1} FW) and chlorophyll b (mg g^{-1} FW) of methi (*Trigonella foenum-graecum*) and lentil (*Lens culanaris*) at 30, 60 and 90 days after sowing.

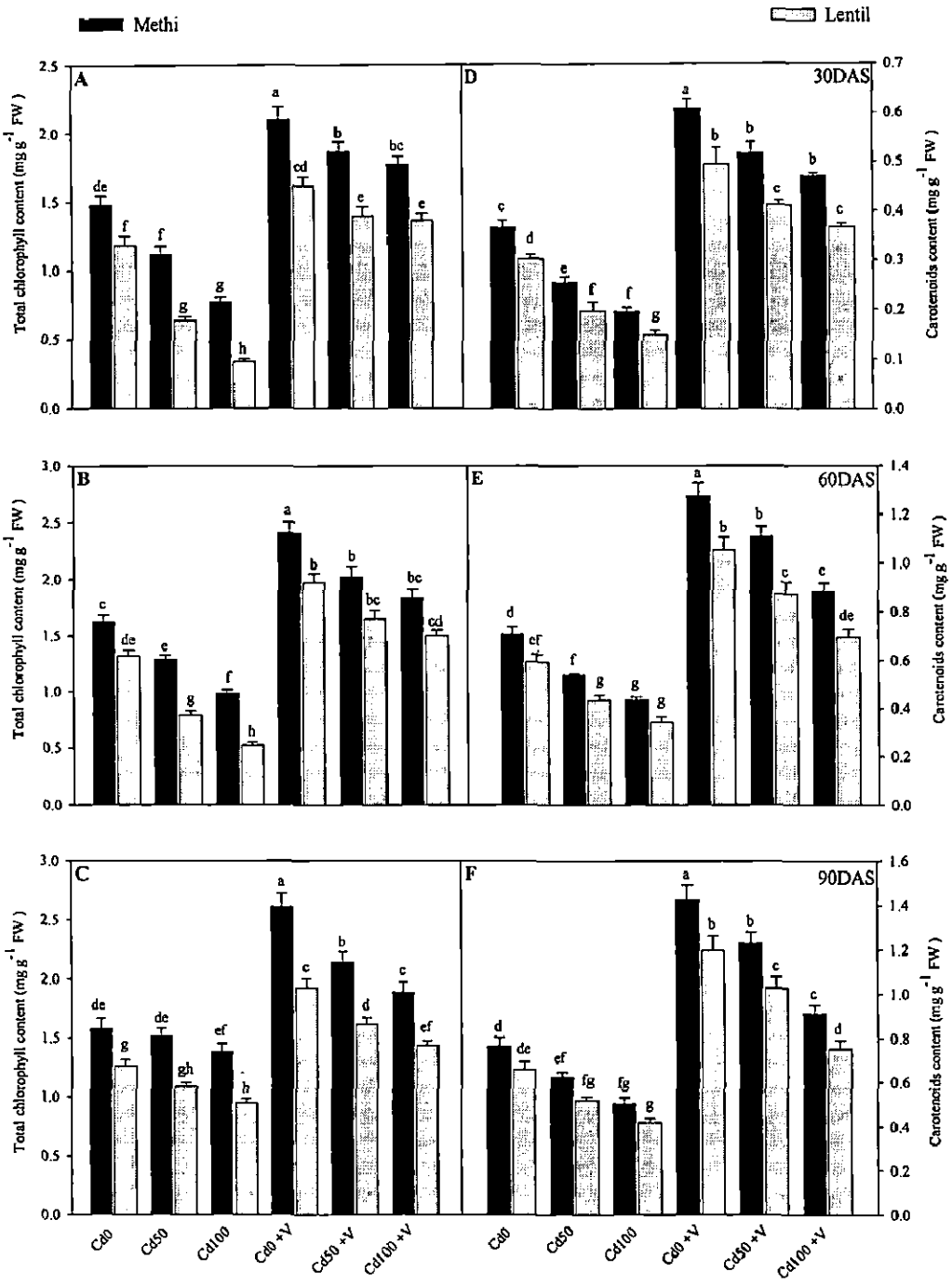


Figure 4.41: Effect of Arbuscular Mycorhizal (AM) fungi application against 0, 50 and 100 mg $CdCl_2$ Kg^{-1} soil on total chlorophyll content (mg g^{-1} FW) and carotenoid content (mg g^{-1} FW) of methi (*Trigonella foenum-graecum*) and lentil (*Lens culanaris*) at 30, 60 and 90 days after sowing.

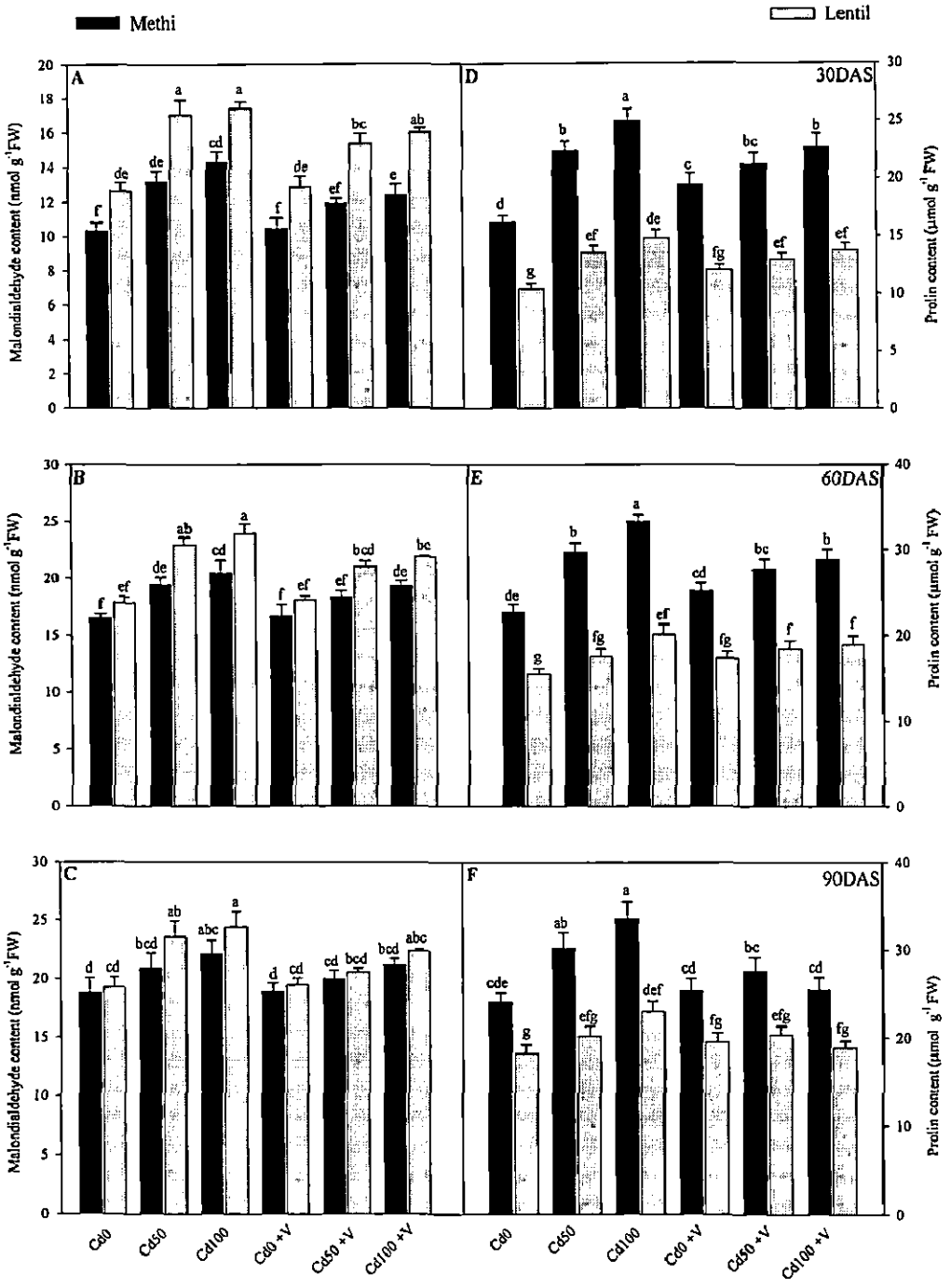


Figure 4.42: Effect of Arbuscular Mycorhizal (AM) fungi application against 0, 50 and 100 mg $CdCl_2$ Kg^{-1} soil on malonaldehyde content (nmol g^{-1} FW) and proline content (µmol g^{-1} FW) of methi (*Trigonella foenum-graecum*) and lentil (*Lens culanaris*) at 30, 60 and 90 days after sowing.

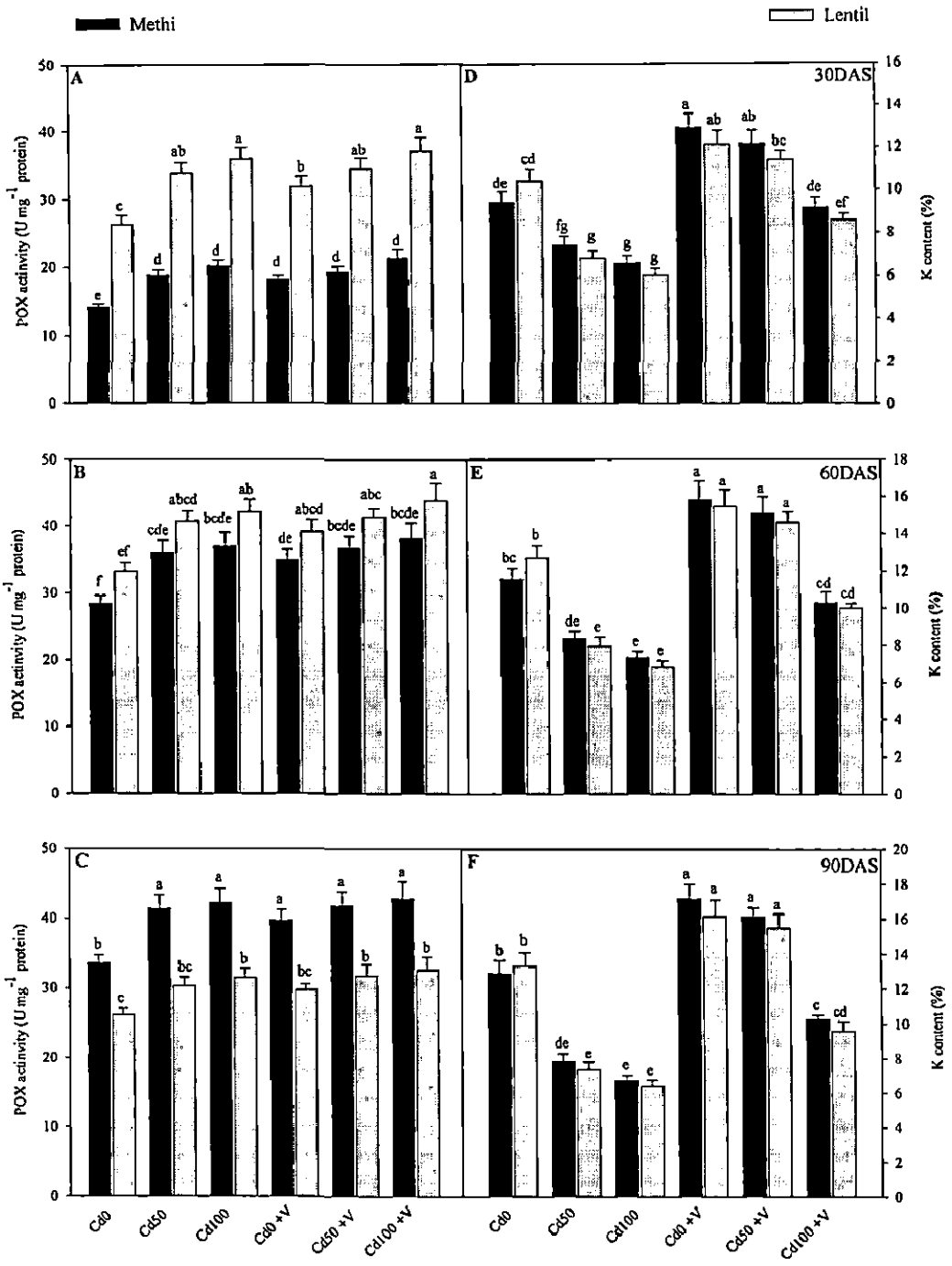


Figure 4.43: Effect of Arbuscular Mycorhizal (AM) fungi application against 0, 50 and 100 mg $CdCl_2$ Kg^{-1} soil on POX activity (U mg^{-1} protein) and potassium content (%) of methi (*Trigonella foenum-graecum*) and lentil (*Lens culanaris*) at 30, 60 and 90 days after sowing.

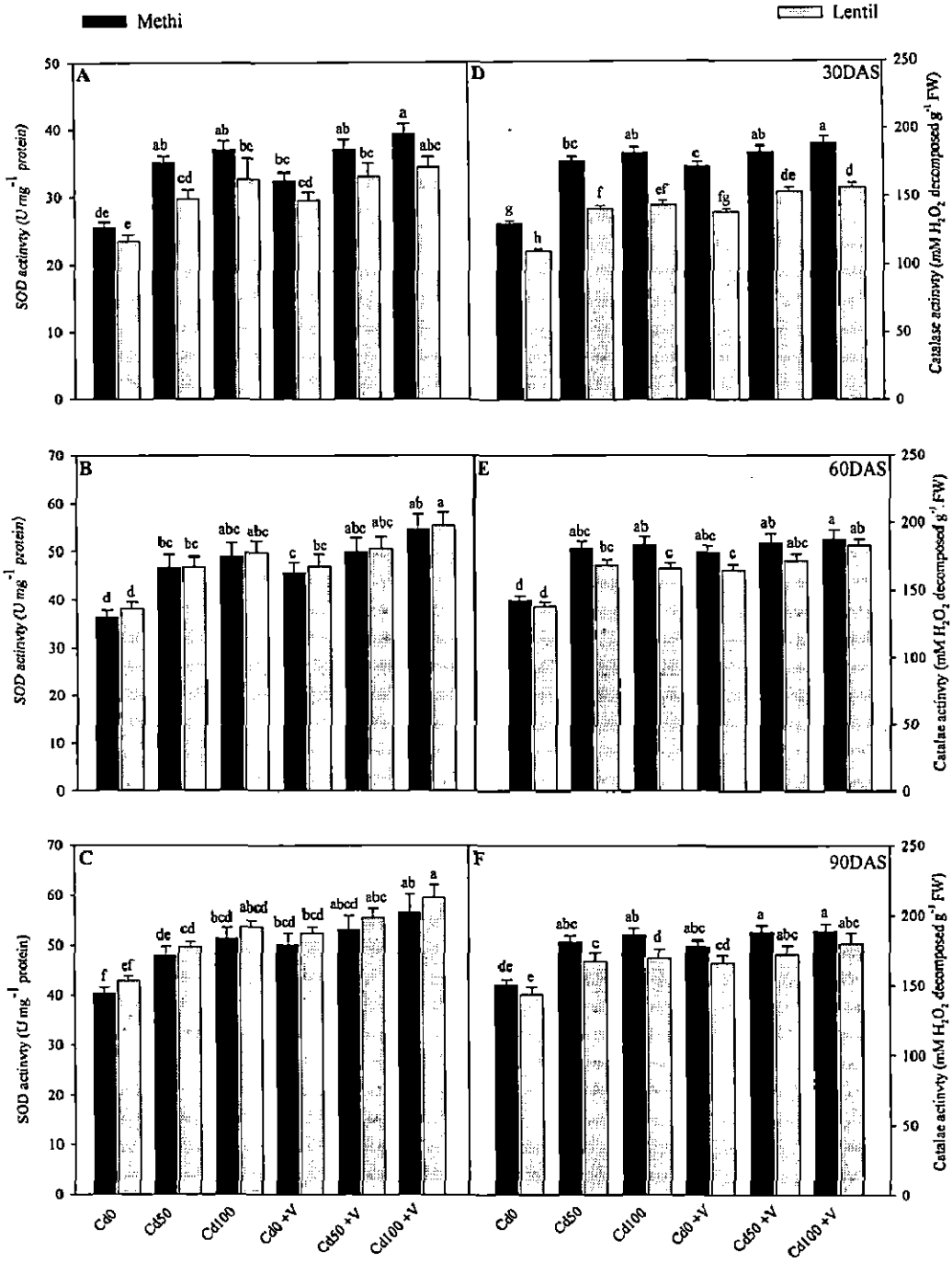


Figure 4.44: Effect of Arbuscular Mycorhizal (AM) fungi application against 0, 50 and 100 mg $CdCl_2$ Kg^{-1} soil on SOD activity (U mg^{-1}protein) and catalase activity (mM H_2O_2 decomposed g^{-1} FW) of methi (*Trigonella foenum-graecum*) and lentil (*Lens culanaris*) at 30, 60 and 90 days after sowing.

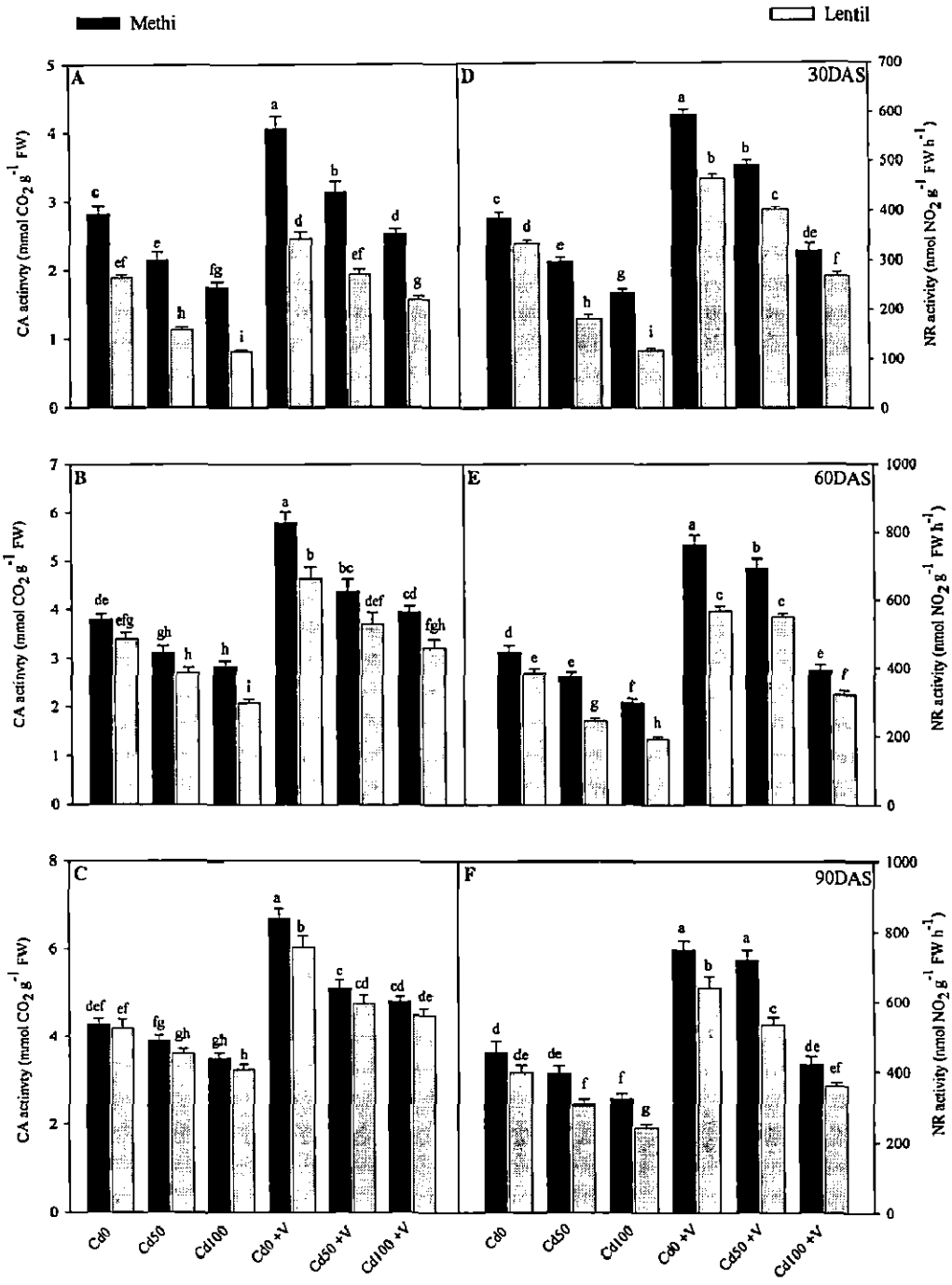


Figure 4.45: Effect of Arbuscular Mycorhizal (AM) fungi application against 0, 50 and 100 mg $CdCl_2$ Kg^{-1} soil on CA activity (mmol CO_2 g^{-1} FW) and NR activity (nmol NO_2 g^{-1}FW h^{-1}) of methi (*Trigonella foenum-graecum*) and lentil (*Lens culanaris*) at 30, 60 and 90 days after sowing.

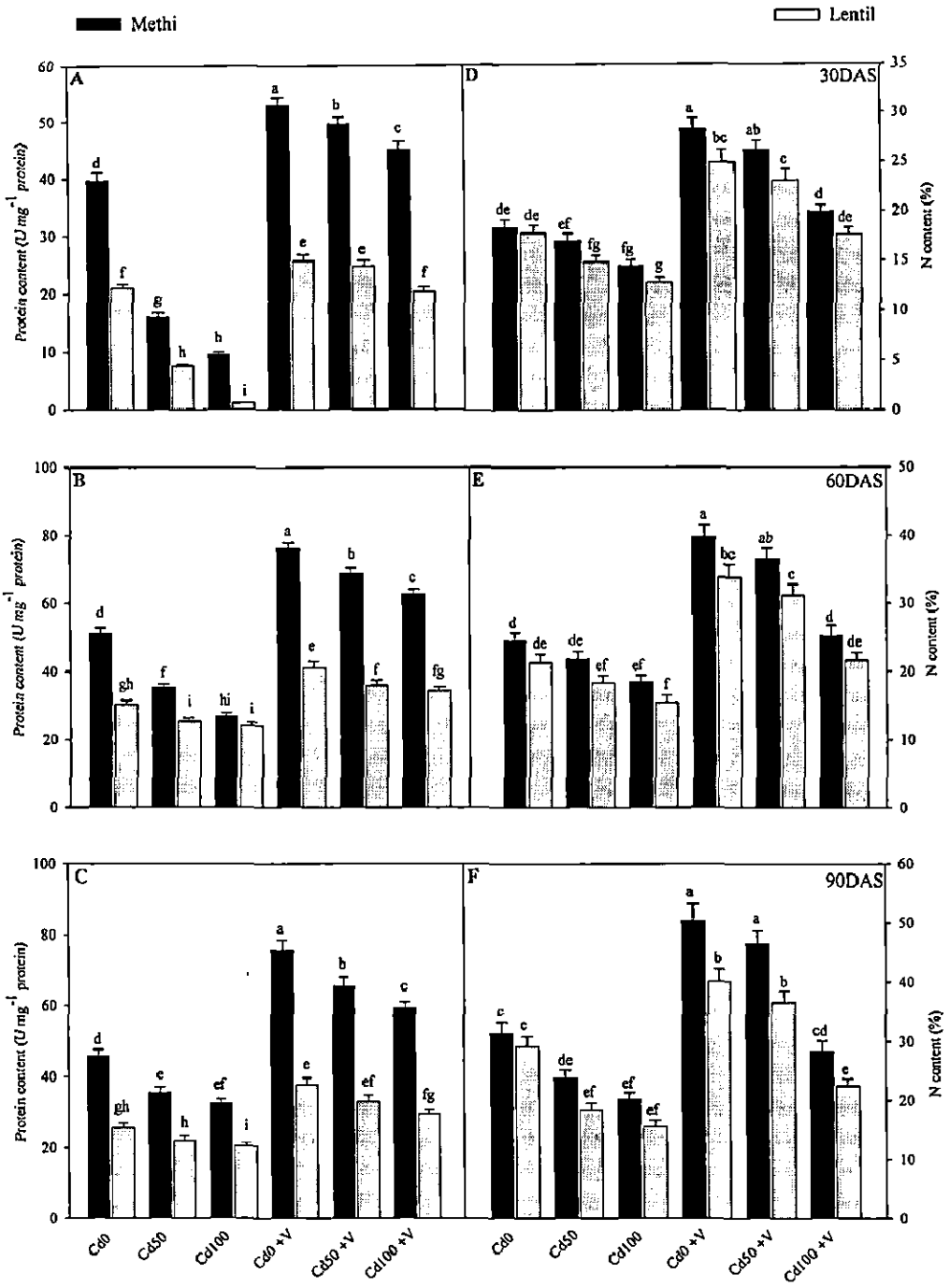


Figure 4.46: Effect of Arbuscular Mycorhizal (AM) fungi application against0, 50 and 100 mg $CdCl_2$ Kg^{-1} soil on protein content (U mgg^{-1} protein) and nitrogen content (%) of methi (*Trigonella foenum-graecum*) and lentil (*Lens culanaris*) at 30, 60 and 90 days after sowing.

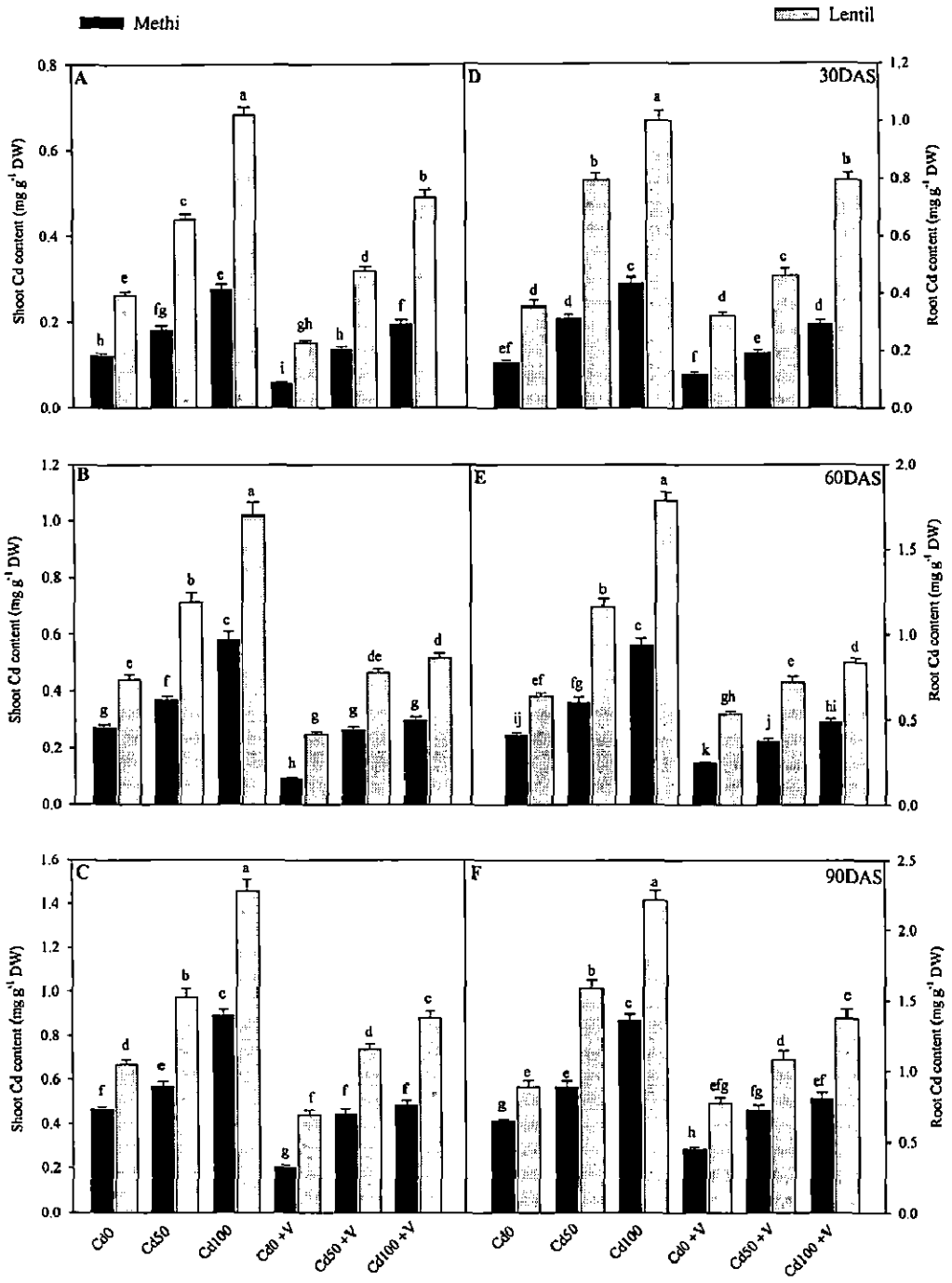


Figure 4.47: Effect of Arbuscular Mycorhizal (AM) fungi application against 0, 50 and 100 mg $CdCl_2$ Kg^{-1} soil on shoot Cd content (µg g^{-1} DW) and root Cd content (µg g^{-1} DW) of methi (*Trigonella foenum-graecum*) and lentil (*Lens culanaris*) at 30, 60 and 90 days after sowing.

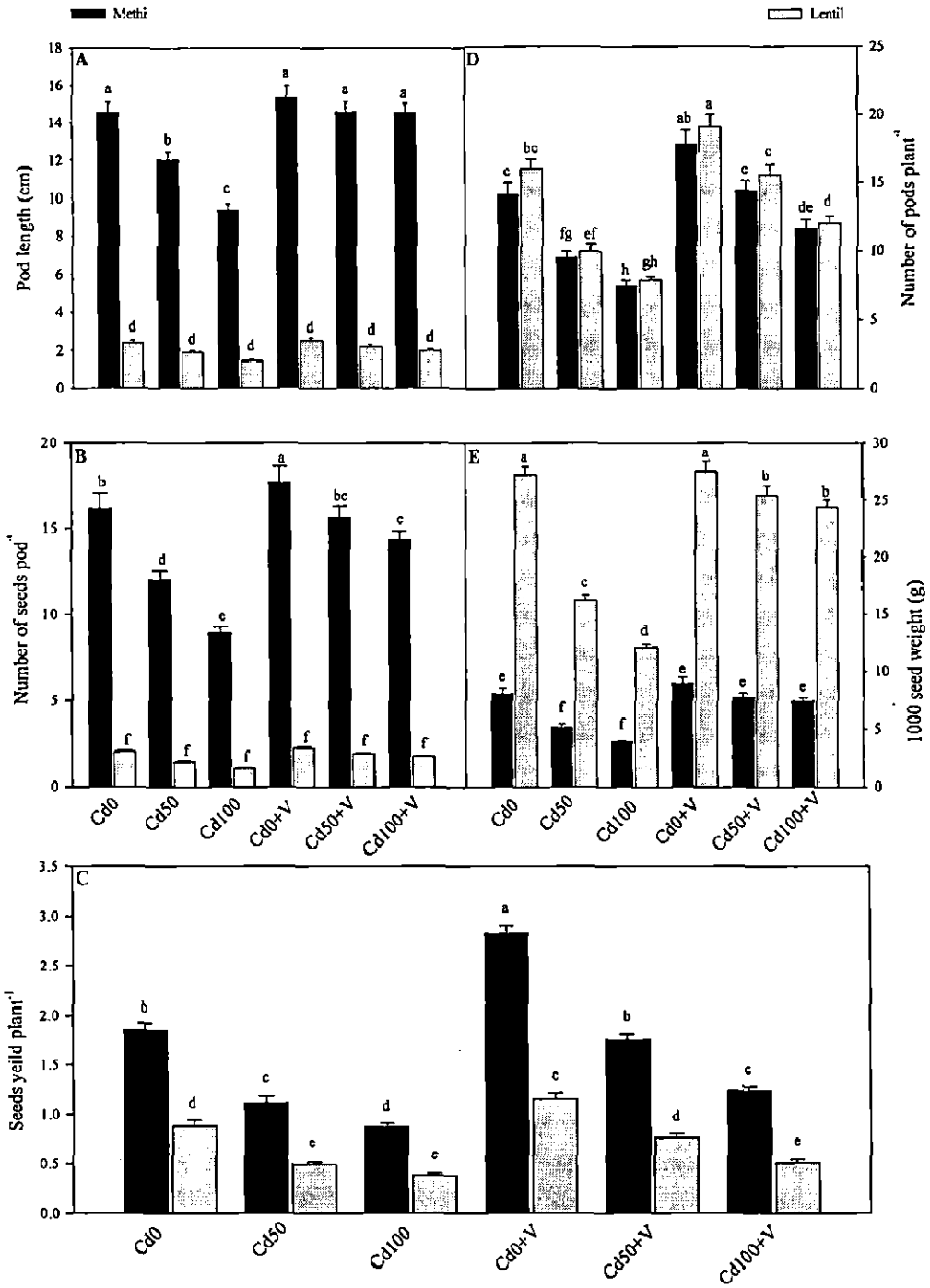


Figure 4.48: Effect of Arbuscular Mycorhizal (AM) fungi application against 0, 50 and 100 mg $CdCl_2$ Kg^{-1} on pod length (cm), number of pods $plant^{-1}$, number of seeds pod^{-1}, 1000 seeds weight and seed yield $plant^{-1}$ of methi (*Trigonella foenum-graecum*) and lentil (*Lens culanaris*) at harvest i.e., 120 days after sowing.

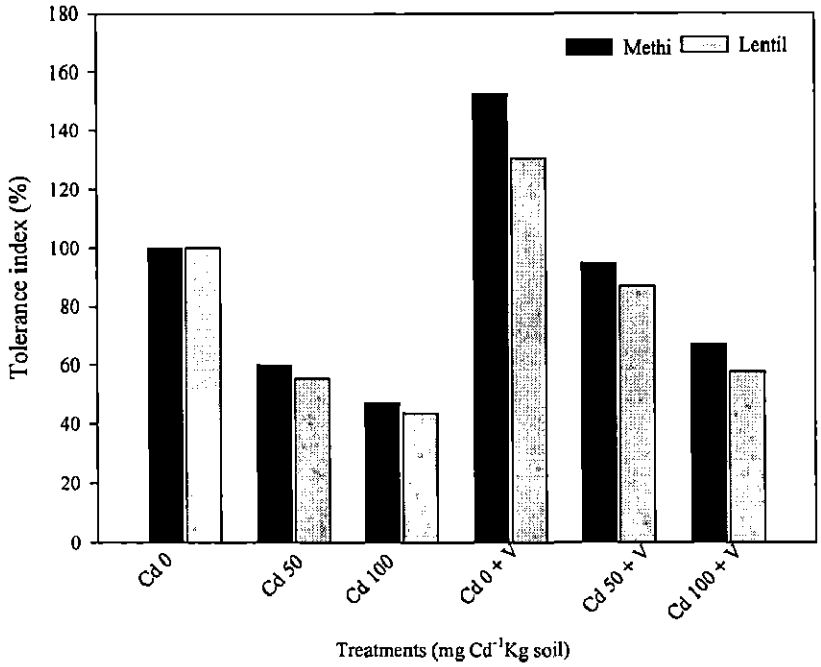


Figure 4.49: Tolerance index of two legumes (methi and lentil) exposed to 0, 50 and 100 mg Cd Kg^{-1} soil alone and seed application with AM fungi. Tolerance index was calculated as per cent change of seed yields with control.

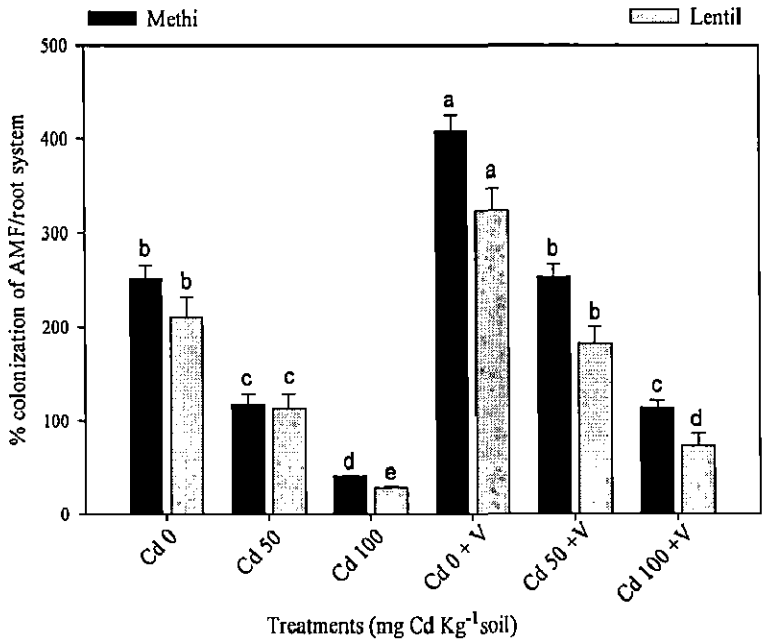


Figure 4.50: Per cent (AM) colonization in roots of two legumes (methi and lentil) exposed to 0, 50 and 100 mg Cd Kg^{-1} soil alone and seed application with AM fungi at 60 days after sowing.

4.3.11 Per cent colonization of AM fungi

Inoculation of AM fungi increased in per cent colonization by 62 and 54% in methi and lentil respectively compared to control at 60 DAS. However, the colonization decreased with the increase doses of Cd (Figure 4.50). However, plants inoculated with AM fungi improved the per cent colonization due to 50 and 100 mg Cd Kg^{-1} soil, as compared to plants subjected with Cd alone.

Summary of Experiment 3

- Soil inoculation of AM fungi effectively reduced the Cd accumulation in root and shoot tissues and its toxic responses in both the legumes. The accumulation of Cd was more in root than shoot. Out of the two legumes, lentil showed more accumulation than methi.
- Soil amended with AM fungi to seeds has high alleviation potential to the effects of Cd-induced reduction in growth, biochemical and yield characteristics of methi and lentil.
- Cd-induced accumulation of MDA content was significantly lowered by AM fungi.
- AM fungi application increased the activities of POX, CAT and SOD of the plants grown in soil treated with Cd. However, the level of activity was different in both the legumes.
- Amendment of AM fungi to soil proved most effective in alleviating the effects 50 mg Cd Kg^{-1} soil.
- Application of AM fungi to soil also lowered the Cd-induced decrease in yield characteristics of both the legumes particularly number of pods and seed yield per plant.
- The application of AM fungi to seeds was found most effective in mitigating the 50 mg Cd Kg^{-1} soil-induced reductions in growth, biochemical and yield characteristics in methi. However, reductions in these characteristics were only lowered in lentil.
- 100 mg Kg^{-1} soil decreased most of the parameters of the two legumes, whereas AM fungi amendment in soil could overcome them only partially in most of the cases.

4.4 Experiment 4: To study the alleviation potential of *Rhizobium* and AM fungi application against Cd-induced effects in Cd-sensitive and Cd non-sensitive legumes

On the similar pattern of Experiment 2 and 3, the Experiment 4 was conducted to study the effectiveness of dual inoculation of *Rhizobium* and AM fungi to methi (Cd least sensitive) and lentil (Cd most-sensitive) against Cd doses (50 mg and 100 mg Kg^{-1} soil) legumes.

The details of results briefly described below and summarized in Figures (4.51 – 4.66)

4.1.1 Growth characteristics

Application of AM fungi and *Rhizobium* alleviated the negative effects on growth parameters (root and shoot length, fresh and dry weight of root and shoot, leaf number per plant and leaf area per plant and number of nodules per root system) of methi and lentil due to Cd amendment (Cd 50 or 100 mg Kg^{-1}) in soil (Figures 4.51- 4.55). The application of both the symbionts completely nullified the adverse effects of 50 mg Cd Kg^{-1} soil for the observed growth parameters and their combined inoculation also mitigated the adverse effect of 100 mg Cd Kg^{-1} soil on growth parameters at all growth stages. Co-inoculation increased all the growth characteristics. However, this increase was more in methi than lentil .The growth improved with the age of the plant (30 to 90 DA). Shoot and root length, root fresh and dry weight, leaf number per plant, leaf area per plant and number of nodules per root system in methi was decreased . Lentil recorded a decrease of 53.5, 35.6% in shoot length; 24.8, 16.2% in root length; 84.6, 44.3% in shoot fresh weight; 24.7, 14.9% in shoot dry weight; 37.9, 53.3% in root dry weight; 26.5, 15.6%; in leaf number 38.7, 32.3% in leaf area; 40.6, 32.5% and 61.1, 37.9% in number of nodules per root system due to 50 and 100 mg Kg^{-1}soil respectively, over the control at 30 DAS. However, dual inoculation with *Rhizobium* and AM fungi almost completely alleviated the negative effects the stress cause due to Cd 50 mg Kg^{-1} soil in all the growth parameters of methi and lentil and the values were at par to that of control plants. Number of nodules per root system, due to co-inoculation of both the symbiont gave a substantial gain of 41.6, 24.9% in methi and 34.4, 16.9% in lentil due to 50 and 100 mg Cd Kg^{-1}soil respectively, over control at 30 DAS.

4.4.2 Photosynthetic attributes

Photosynthetic pigments (chlorophyll a, chlorophyll b, total chlorophyll and carotenoid contents) in the leaves of two legumes decreased with the increase in the level of Cd in soil. However, this decrease was more pronounced in lentil as compared to methi (Figures 4.56 - 4.57). Co-inoculated plants completely overcome the decline of photosynthetic pigments content due to Cd stress. Dual inoculated plants showed an increase of 60.8, 54.5% in chlorophyll a; 67.6, 55.1% in chlorophyll b; 62.8, 54.7% in total chlorophyll and 97.5, 90.5% in carotenoids content in methi and lentil respectively, over the control due to 50 mg Cd Kg^{-1}soil at 90 DAS. Moreover, dual inoculated plants showed an increase of 22.1, 17.8% in total chlorophyll and, 4.0, 9.9% in carotenoid content in methi and lentil respectively, over the control at 90 DAS.

4.4.3 Metabolic stress markers: Lipid peroxidation and proline content

MDA and proline content increased with the age of the plants (Figure 4.58). These parameters increase with the increase in the level of Cd in soil but lentil showed more accumulation than methi. Dual inoculated plants decreased MDA and proline contents at all growth stages. Lentil showed an increase of 47.1, 35.7% in MDA 42.3 32.7% in proline due to 50 or 100 mg Cd Kg^{-1}soil respectively, over the control at 30 DAS.

4.4.4 Enzymatic stress markers: Antioxidant enzymes activity

Activity of POX, CAT and SOD in the leaves of methi and lentil increased as denoted by their values (Figures 4.59 - 4.60). The response of antioxidant enzymes was differential to Cd doses in methi and lentil and their activities were enhanced with the increase in the level of Cd in soil. An increase of 26.9, 45.3% in POX and 32.4, 41.0% in SOD was observed in lentil and methi respectively, over the control at 30 DAS due to 100 mg Cd Kg^{-1} soil. Co- inoculated plants showed further increase of POX, CAT and SOD activity. Dual inoculation of both the symbionts caused an increase of 37.9, 48.55% in POX; 42.9, 48.13% in CAT and 42.1, 50.9% in SOD in methi and lentil respectively compared to control at 30 DAS due to100 mg Cd Kg^{-1} soil.

4.4.5 Carbonic anhydrase and nitrate reductase activities

The activity of CA, NR enzymes and protein content decreased with the increase in the level of Cd in soil. The extent of decrease was more in lentil than methi (Figures

4.61 - 4.62). Dual inoculation enhanced these parameters and also lowered reduction in them caused by 50 mg Cd Kg^{-1}soil However, it could not alleviate completely the reduction in these parameters caused by 100 mg Cd Kg^{-1}soil. The NR and CA activity showed a decline of 21.7, 22.9% in methi and 44.6, 38.6% in lentil due to 50 mg Cd Kg^{-1}soil respectively, as compared to control at 30 DAS.

4.4.6 Leaf protein content

The leaf protein content also followed the similar trend for treatments as that of the enzymes. However, it was comparatively higher in *Rhizobium* inoculated plants compared to non-inoculated plants supplemented with 50 or 100 mg Cd Kg^{-1} soil (Figure 4.31).The per cent increase in protein content of leaf was highest at 60 DAS as compared to early or later growth stages. Enzyme activity and protein content in methi reflected higher values than lentil. Addition of Cd decreased the protein content. However, this decrease was more with 100 mg Cd Kg^{-1} soil. A decline of 63.0 and 76.5% in methi and lentil respectively, as compared to control due to 50 mg Cd Kg^{-1} soil at 30 DAS. Plants inoculated with *Rhizobium* showed increase of 37.1 and 21.4% in both the legumes due to 50 mg Cd Kg^{-1} soil at 60 DAS as compared to control.

4.4.7 N, P and K content in leaves

Cadmium treatment significantly decreased the leaf N, P and K content with the increase in the level of Cd in soil at all growth stages. Moreover, this decline was more in lentil than methi (Figures 4.62 - 4.59). The decrease of N and P content was insignificant in plants treated with 50 mg Cd Kg^{-1}soil but it was significant in plants supplemented with 100 mg Cd Kg^{-1}soil. The decrease of N, P and K content was 18.1, 25.6%; 16.2, 18.3%; and 25.1, 39.4% in methi and lentil respectively, over the control at 30 DAS due to 100 mg Cd Kg^{-1}soil. Dual inoculation of symbiont caused an increase of 93.1,81.9; 52.2,86.1 and 40.5,29.4 % in N, P and K in methi and lentil, respectively, over the control at 90 DAS. Co-inoculated plants significantly increased the nutrient accumulation in plants supplemented with Cd but the extent of increase was more for 50 mg Cd Kg^{-1}soil.

4.4.8 Cadmium accumulation in shoot and root

Cd accumulation in shoot and root increased with the increasing dose and also with age in both the legumes (Figure 4.63). Lentil showed more accumulation than methi,

whereas, root accumulated more Cd than shoots in both the plants. In methi, accumulation of Cd in root and shoot was 0.9, 0.6 μg g^{-1} dry weight due to 50 mg Cd Kg^{-1} soil due to 100 mgCd Kg^{-1} it was 1.3, 0.9 μg g^{-1} dry weight at 90 DAS. *Rhizobium* and AM fungi maximally lowered the accumulation of Cd in plants supplemented with Cd. Root and shoot gave an accumulation of 0.5, 0.8 and 0.4, 0.5μg g^{-1} dry weights soil in lentil and methi respectively, over the control due to 100 mg Cd Kg^{-1} at 90 DAS. Dual inoculated plants accumulated minimum amount of Cd in root and shoot in control plants. (DATA)

4.4.9 Yield characteristics

Cadmium treatments significantly decrease the yield characteristics (pod length, number of pods per plant, number of seeds per pod, seed yield per plant, 1000 seed weight) at harvest and the extent of decrease was greater in lentil than methi (Figure 4.64). Supplementations of 100 mg Cd Kg^{-1} soil gave a reduction of 46.5, 50.9% in number of pods per plant and 50.6, 55.4% in seed yield in methi and lentil respectively, as compared to the control at 120 DAS. Dual inoculation almost completely lowered the Cd-induced (50 mg Kg^{-1} soil) reduction in yield characteristics of both the plants. However, synergistic interaction with the microbes only partially alleviated the stress caused due to 100 mg Cd Kg^{-1} soil. Dual inoculated plants showed a substantial gain of 8.1, 5.4% in number of pods per pants and 4.3, 4.2% in yield per plant in methi and lentil respectively, as compared to control. Maximum yield were found control plants inoculated with symbionts.

4.4.10 Tolerance index

The tolerance index of legumes was calculated in terms of decrease in yield with the increasing doses of Cd. Methi showed highest tolerance followed by broad bean, chick pea, pea whereas lentil emerged as the least tolerant. Inoculation of both the microbes increased the tolerance of both the plants but this increase was more in methi than lentil (Figure 4.65). However, methi showed more tolerance than lentil. Dual inoculation caused an increase of 23.8 and 34.7% in methi and lentil respectively due to 100 mg Cd Kg^{-1}.

4.4.11 Per cent colonization of AM fungi

The per cent AM colonization increased by 93 and 83% in two legumes with the inoculation of PGPMs i.e. AM fungi and *Rhizobium*. With the increasing Cd content

(0, 50 or 100 mg Kg^{-1} of soil) the per cent colonization of AM fungi, however, decreased. AM colonization was absent (0%) in the methi and lentil plants which were not inoculated with PGPMs and three Cd contents (Figure 4.66). However, plants inoculated with both the symbionts improved the per cent colonization due to 50 and 100 mg Cd Kg^{-1} soil as compared to plants amended with Cd alone.

Summary of Experiment 4

- Soil inoculation of symbionts effectively reduced the Cd accumulation in root and shoot tissues and its toxic responses in both the legumes. The accumulation of Cd was more in root than shoot.. Out of the two legumes, lentil showed more accumulation than methi
- Soil amended with microbes has high alleviation potential to the effects of Cd - induced reduction in growth, biochemical and yield characteristics of methi and lentil.
- Cd-induced accumulation of MDA content was significantly lowered by the application of microbes.
- AM and *Rhizobium* application increased the activities of POX, CAT and SOD of the plants grown in soil treated with Cd. However, the level of activity was different in both the legumes.
- Amendment of AM fungi and *Rhizobium* to soil proved most effective in alleviating the effects 50 mg Cd Kg^{-1} soil.
- Application of AM fungi to the soil also lowered the Cd-induced decrease in yield characteristics of both the legumes particularly number of pods and seed yield per plant.
- Application of *Rhizobium* and AM to soil improved the number of nodules per root system which strengthened the NR activity and N content plants treated with Cd 50 mg Kg^{-1} soil treated plants.

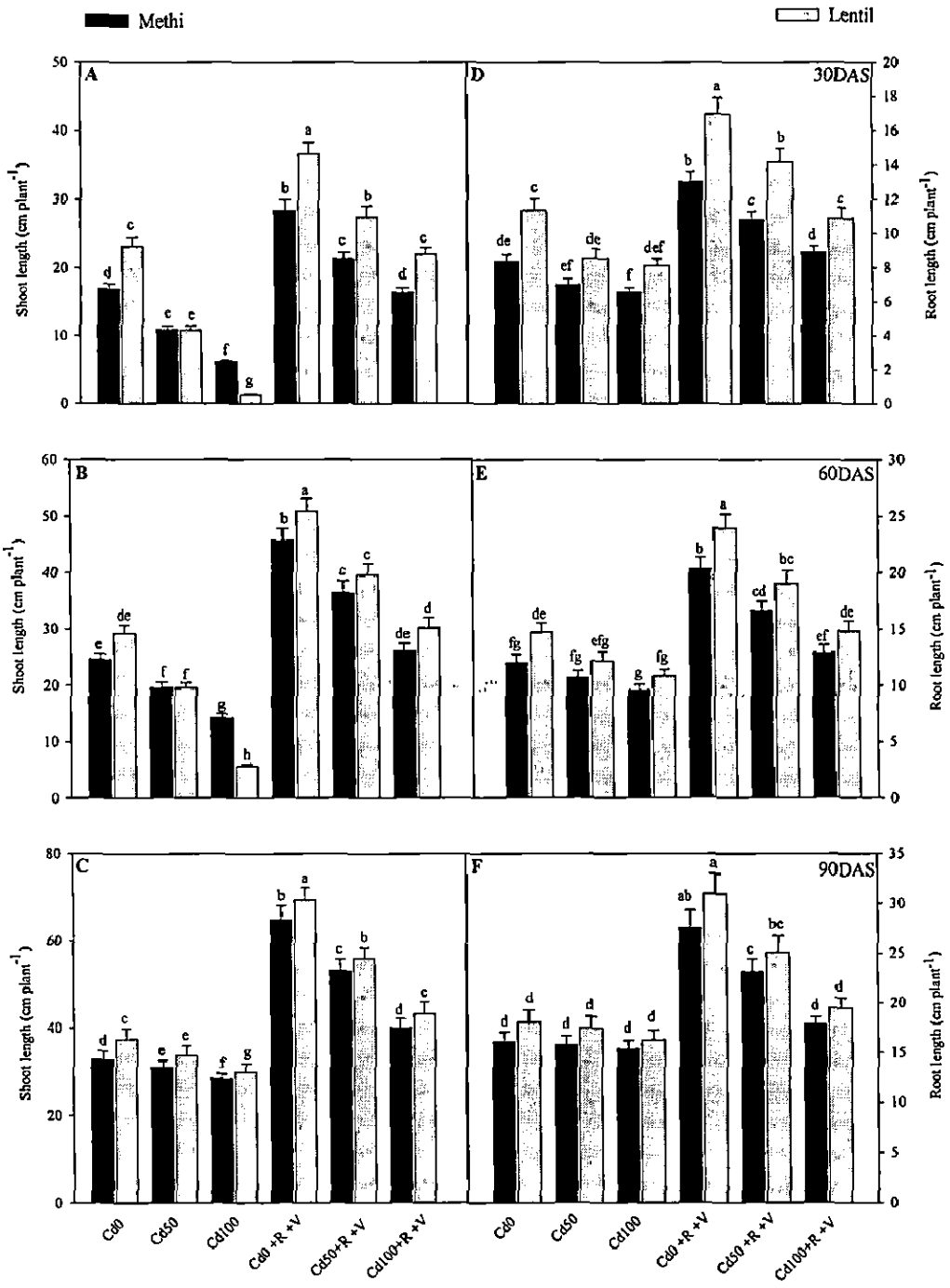


Figure 4.51: Effect of *Rhizobium* (R) inoculation and Arbuscular Mycorhizal (AM) fungi application against 0, 50 and 100 mg Cd Kg^{-1} soil on shoot length and root length (cm) of methi (*Trigonella foenum-graecum*) and lentil (*Lens culanaris*) at 30, 60 and 90 days after sowing.

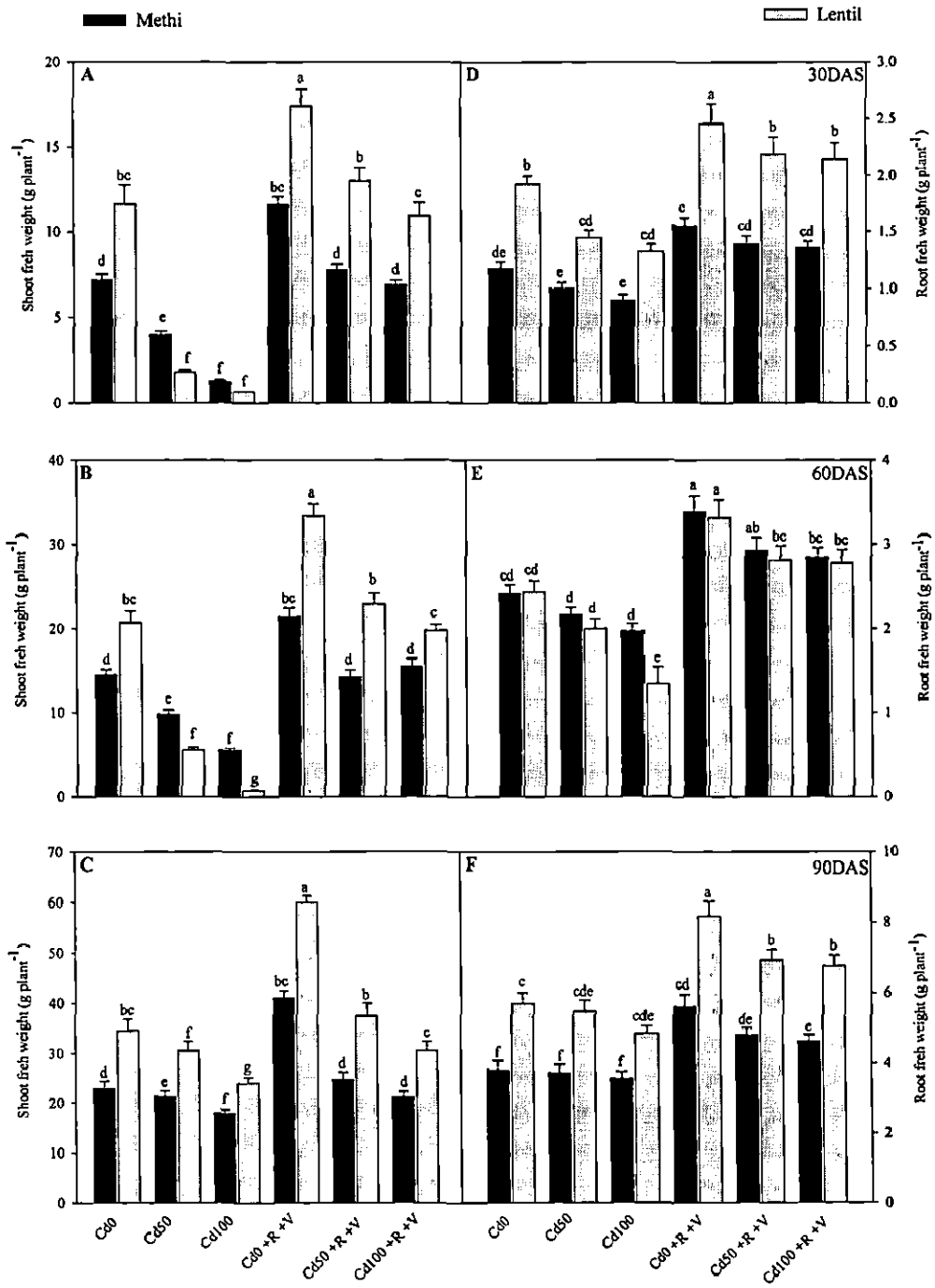


Figure 4.52: Effect of *Rhizobium* (R) inoculation and Arbuscular Mycorhizal (AM) fungi application against 0, 50 and 100 mg Cd Kg^{-1} soil on shoot fresh weight and root fresh weight (g $plant^{-1}$) of methi (*Trigonella foenum-graecum*) and lentil (*Lens culanaris*) at 30, 60 and 90 days after sowing.

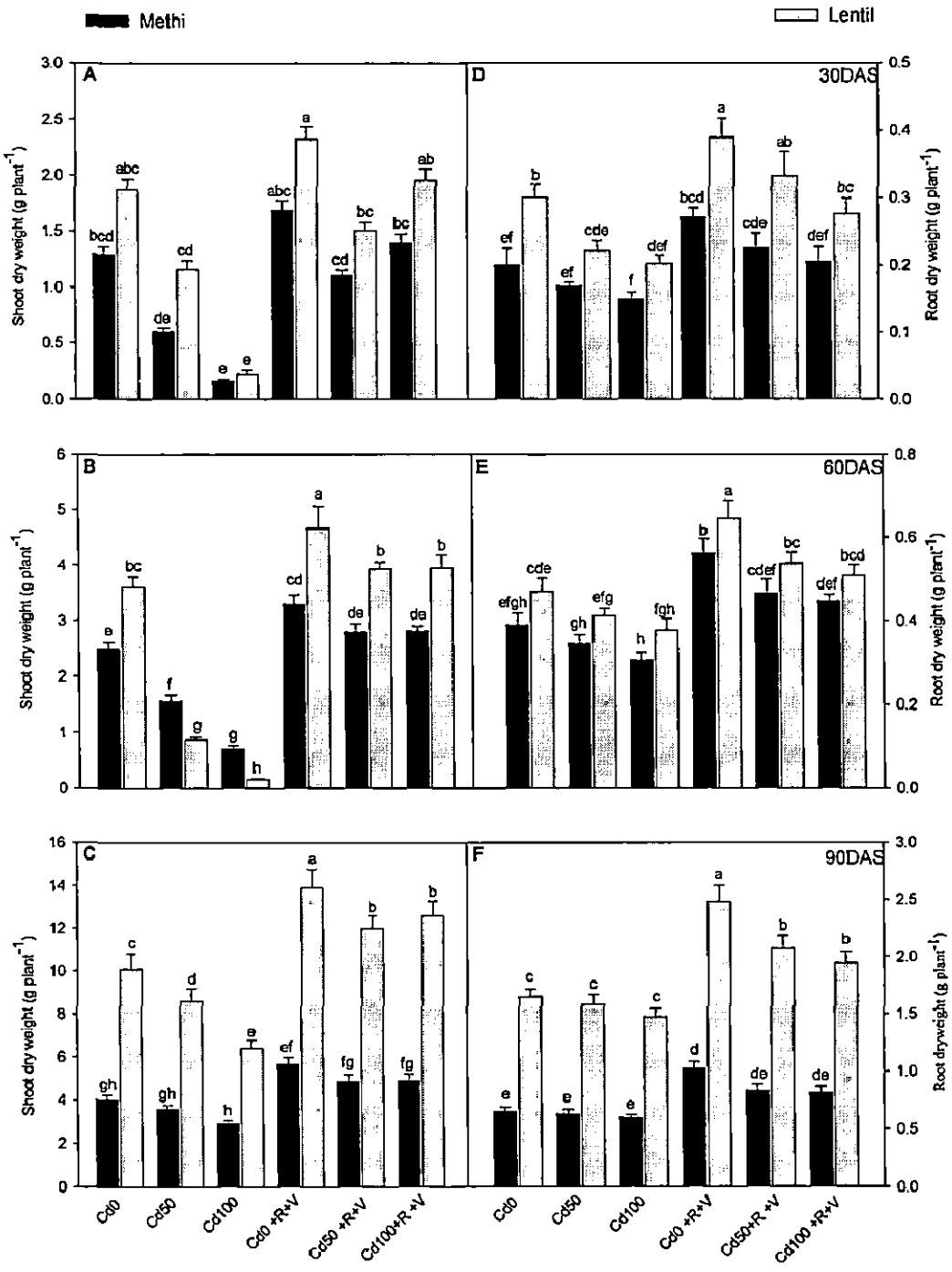


Figure 4.53: Effect of *Rhizobium* (R) inoculation and Arbuscular Mycorhizal (AM) fungi application against 0, 50 and 100 mg Cd Kg^{-1} soil on shoot dry weight and root dry weight (g $plant^{-1}$) of methi (*Trigonella foenum-graecum*) and lentil (*Lens culanaris*) at 30, 60 and 90 days after sowing.

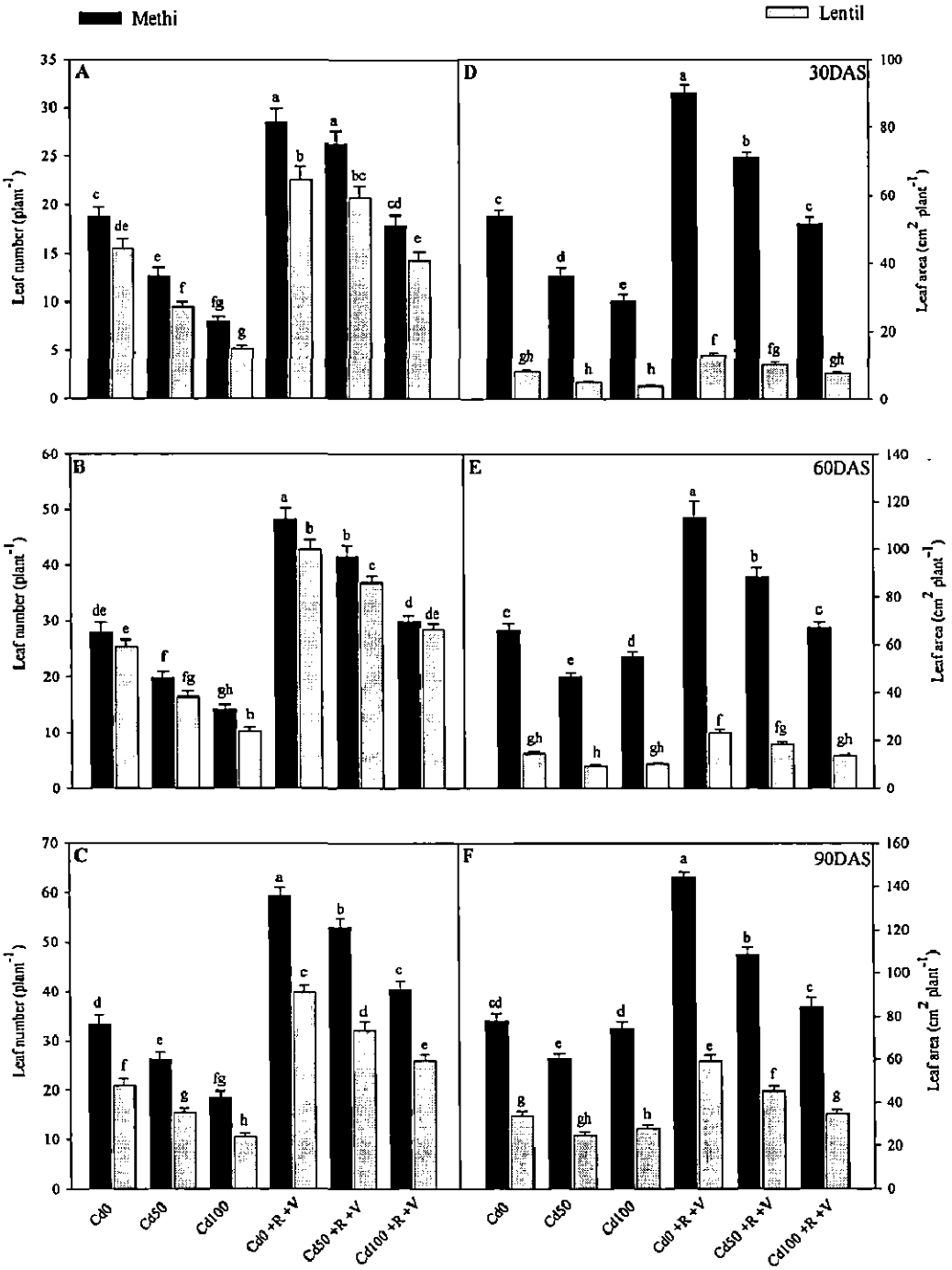


Figure 4.54: Effect of *Rhizobium* (R) inoculation and Arbuscular Mycorhizal (AM) fungi application against 0, 50 and 100 mg Cd Kg^{-1} soil on leaf number ($plant^{-1}$) weight and leaf area (cm^2 $plant^{-1}$) of methi (*Trigonella foenum-graecum*) and lentil (*Lens culanaris*) at 30, 60 and 90 days after sowing.

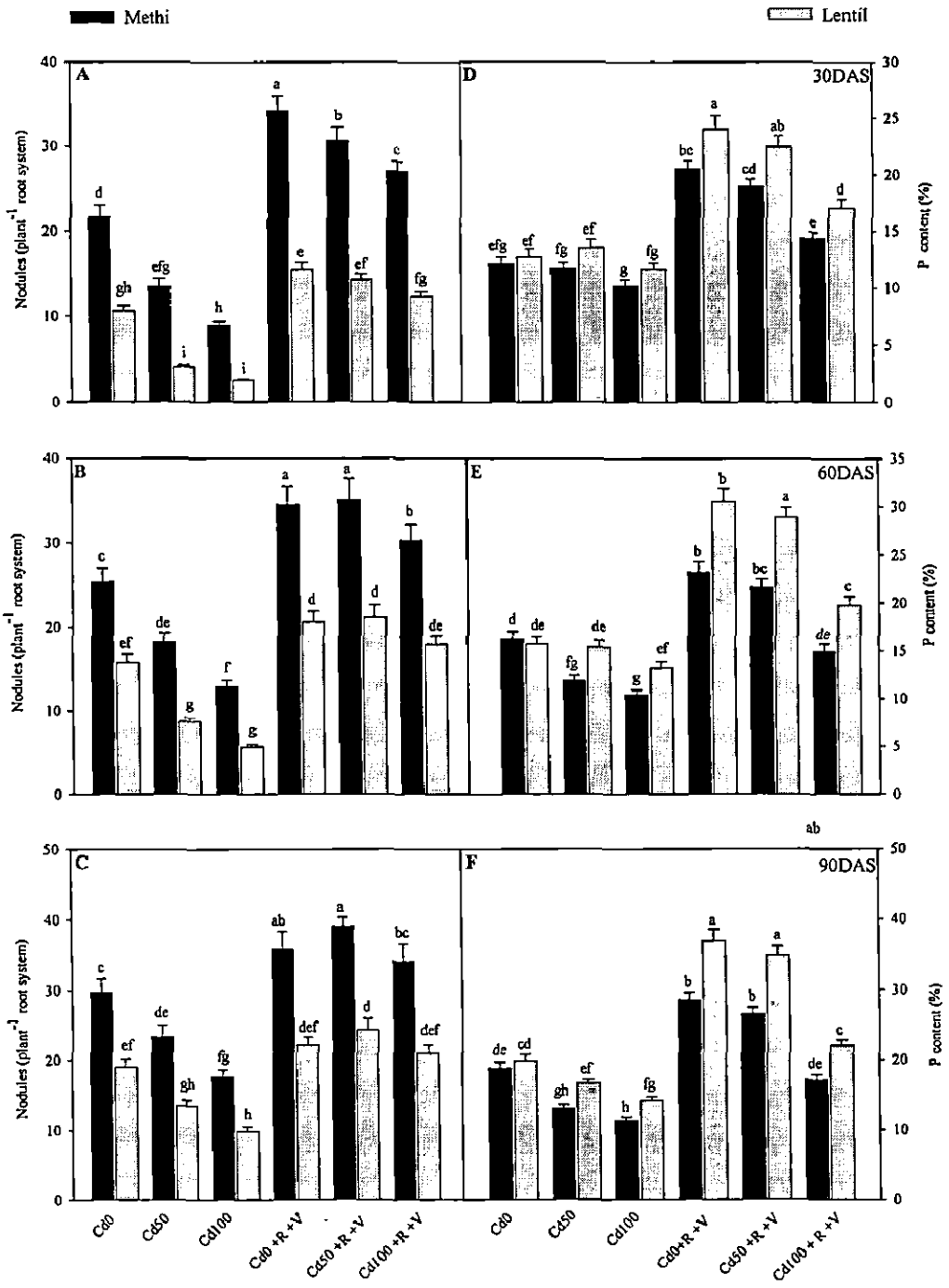


Figure 4.55: Effect of *Rhizobium* (R) inoculation and Arbuscular Mycorhizal (AM) fungi application against 0, 50 and 100 mg Cd Kg^{-1} soil on nodules ($root^{-1}$ system) weight and phosphorous content (%) of methi (*Trigonella foenum-graecum*) and lentil (*Lens culanaris*) at 30, 60 and 90 days after sowing.

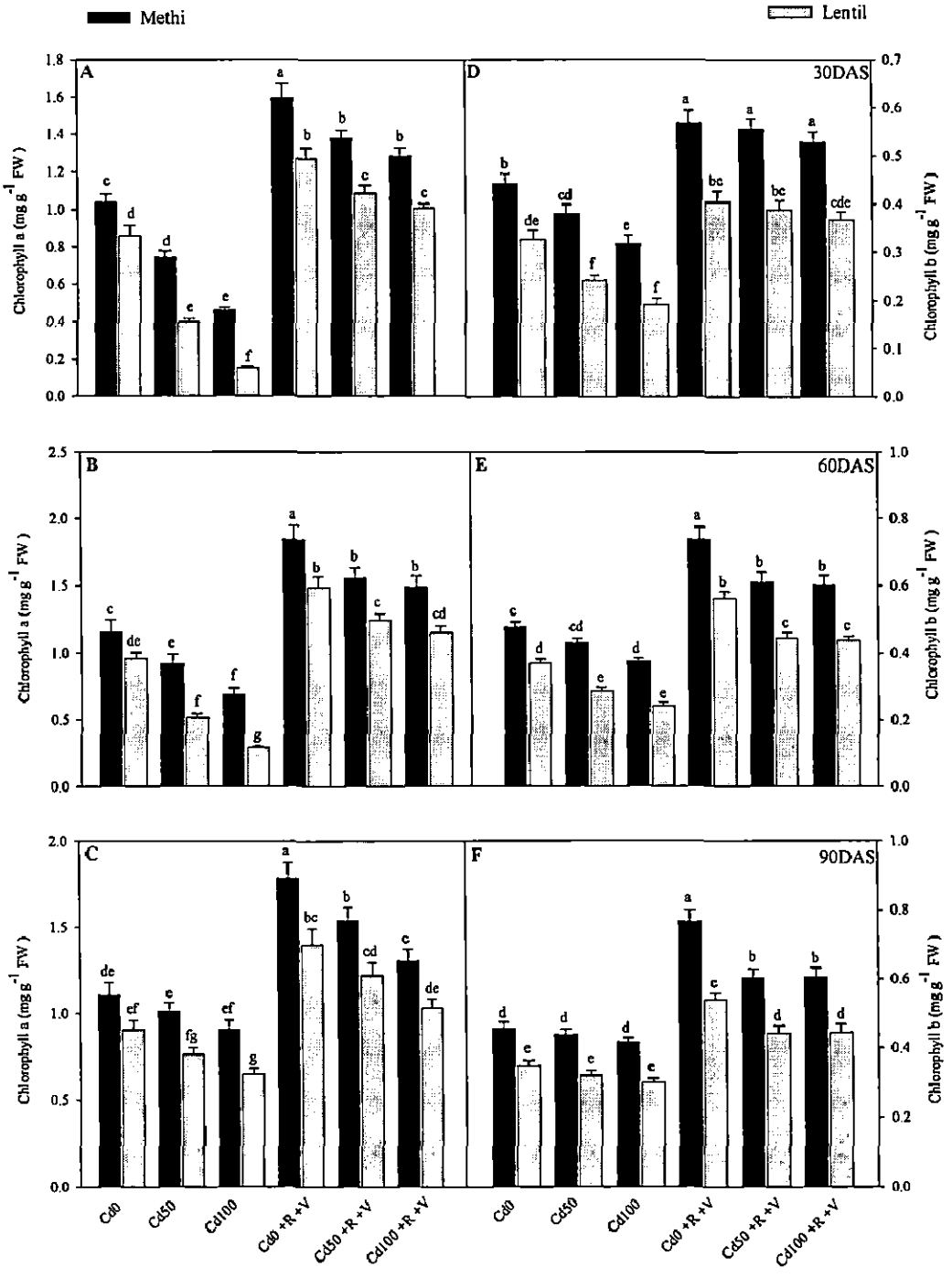


Figure 4.56: Effect of *Rhizobium* (R) inoculation and Arbuscular Mycorhizal (AM) fungi application against 0, 50 and 100 mg Cd Kg^{-1} soil on chlorophyll a (mg g^{-1} FW) weight and chlorophyll b (mg g^{-1} FW) of methi (*Trigonella foenum-graecum*) and lentil (*Lens culanaris*) at 30, 60 and 90 days after sowing.

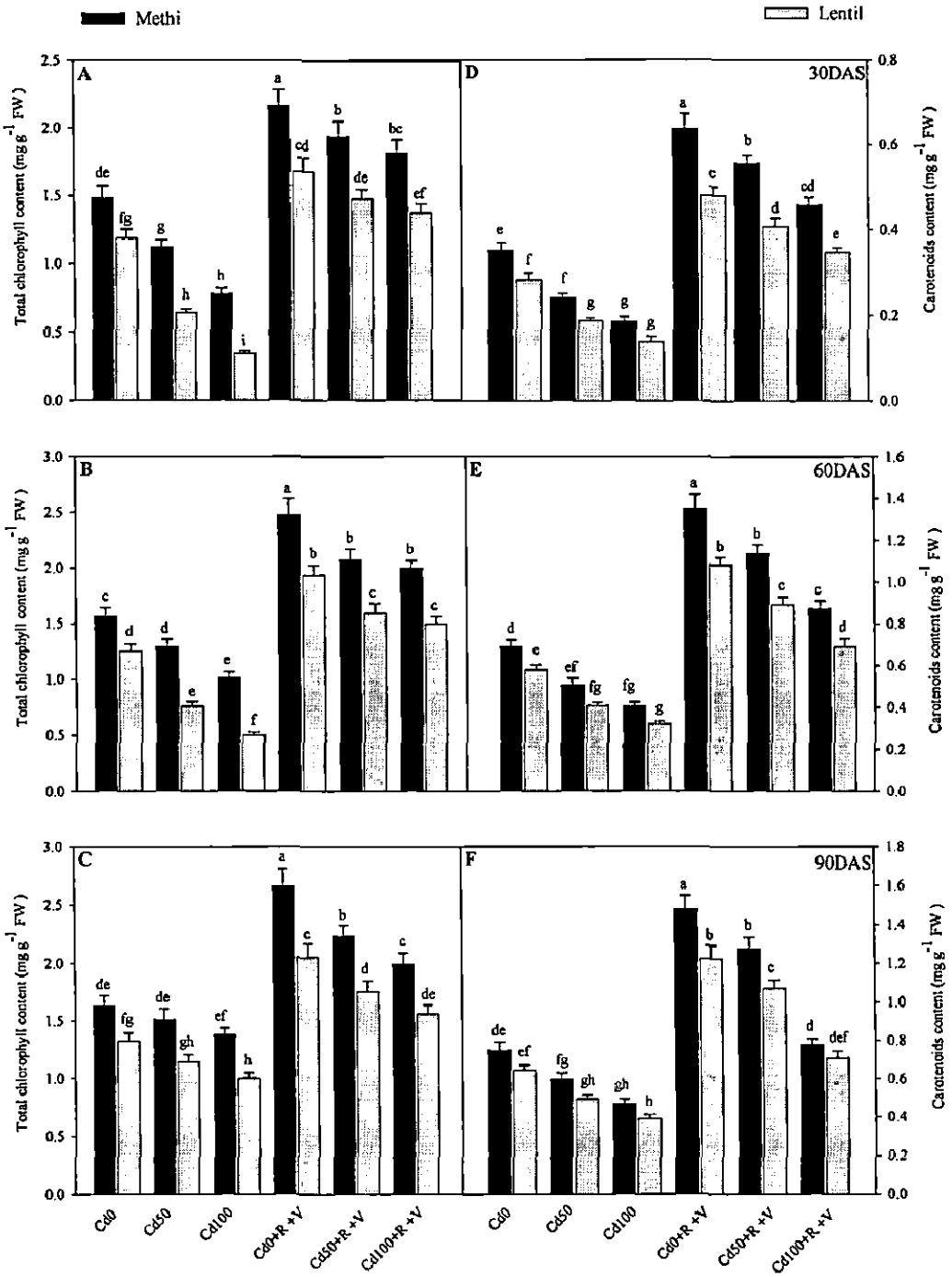


Figure 4.57: Effect of *Rhizobium* (R) inoculation and Arbuscular Mycorhizal (AM) fungi application against 0, 50 and 100 mg Cd Kg^{-1} soil on total chlorophyll content (mg g^{-1} FW) weight and carotenoid content (mg g^{-1} FW) of methi (*Trigonella foenum-graecum*) and lentil (*Lens culanaris*) at 30, 60 and 90 days after sowing.

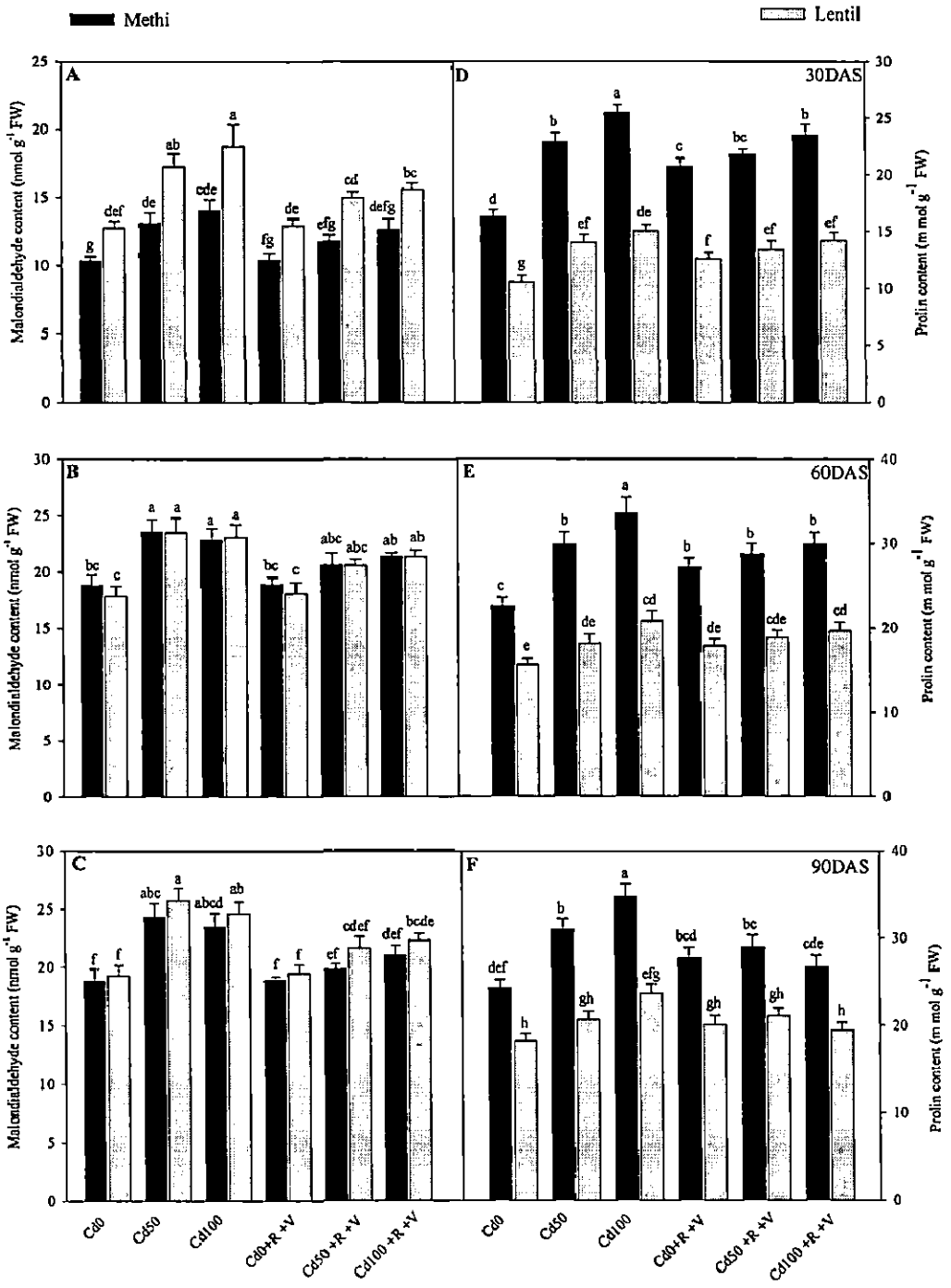


Figure 4.58: Effect of *Rhizobium* (R) inoculation and Arbuscular Mycorhizal (AM) fungi application against 0, 50 and 100 mg Cd Kg^{-1} soil on malondialdehyde level (nmol g^{-1} Fw) weight and prolin content (µmol g^{-1} FW) of methi (*Trigonella foenum-graecum*) and lentil (*Lens culanaris*) at 30, 60 and 90 days after sowing.

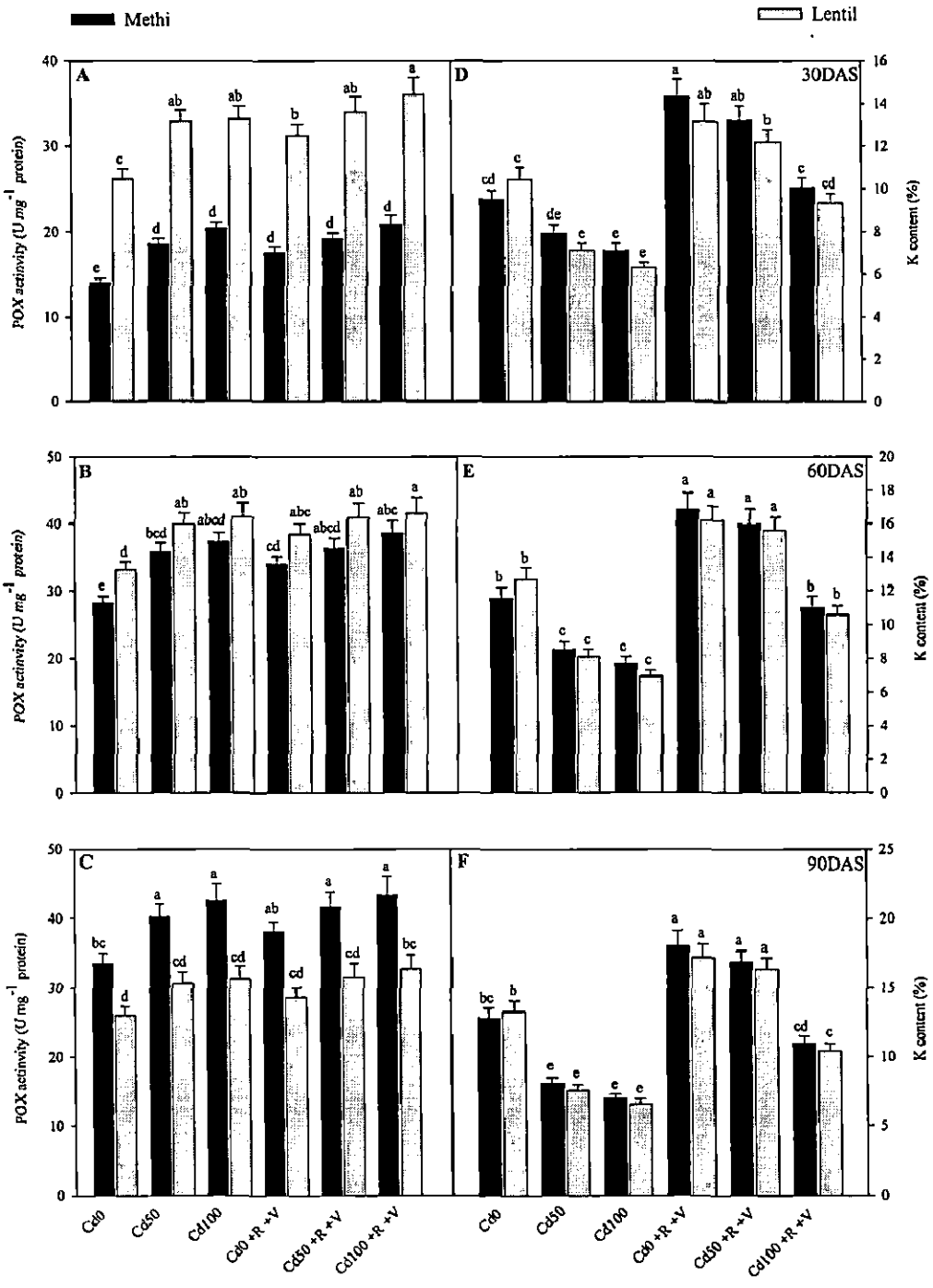


Figure 4.59: Effect of *Rhizobium* (R) inoculation and Arbuscular Mycorhizal (AM) fungi application against 0, 50 and 100 mg $CdCl_2$ Kg^{-1} soil on peroxidase (U mg^{-1} protein) activity and potassium (%) content of methi (*Trigonella foenum-graecum*) and lentil (*Lens culanaris*) at 30, 60 and 90 days after sowing.

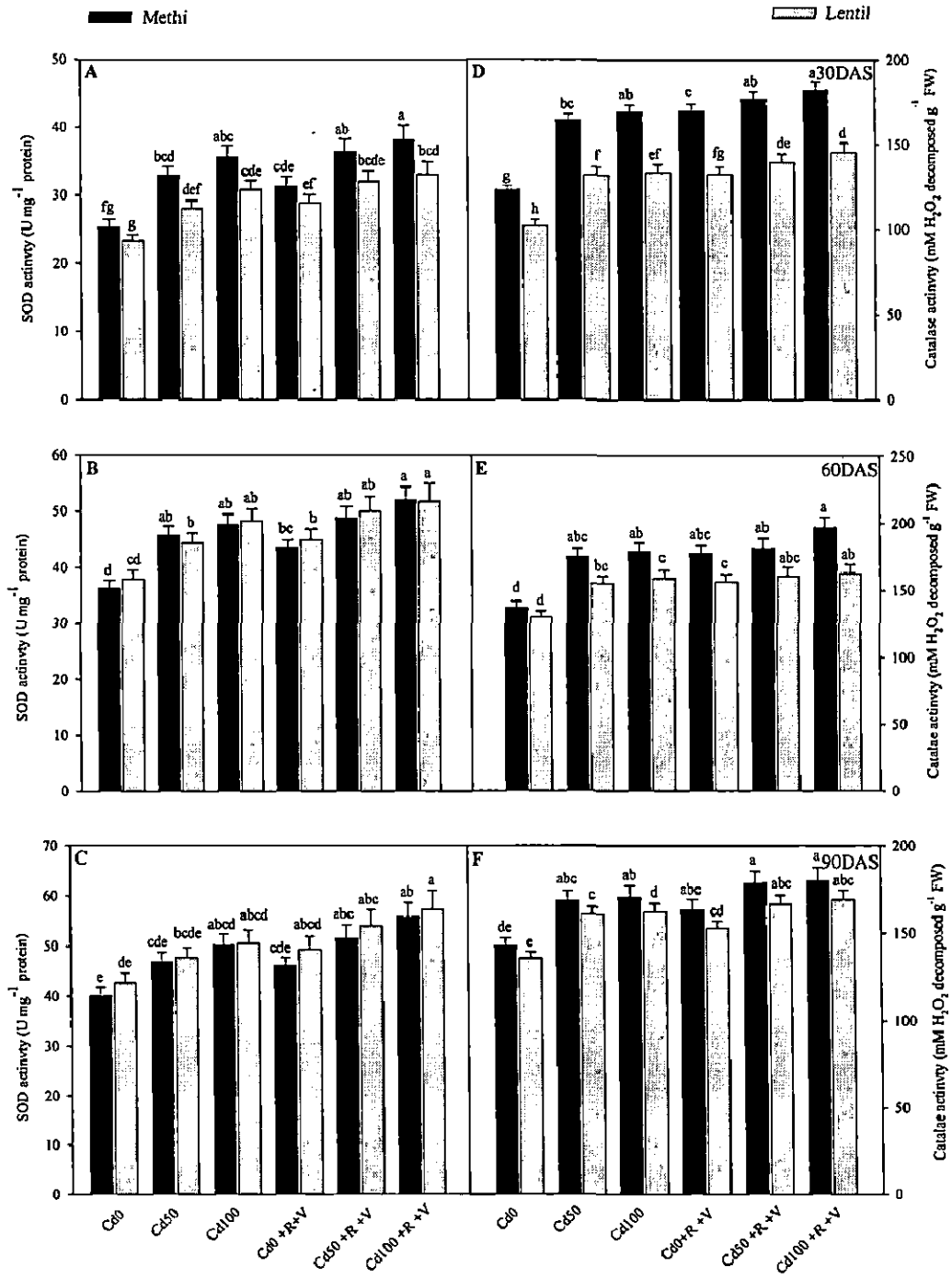


Figure 4.60: Effect of *Rhizobium* (R) inoculation and Arbuscular Mycorhizal (AM) fungi application against 0, 50 and 100 mg $CdCl_2$ Kg^{-1} soil on SOD activity (U mg^{-1}protein) and catalase activity (mM H_2O_2 decomposed g^{-1}FW) of methi (*Trigonella foenum-graecum*) and lentil (*Lens culanaris*) at 30, 60 and 90 days after sowing.

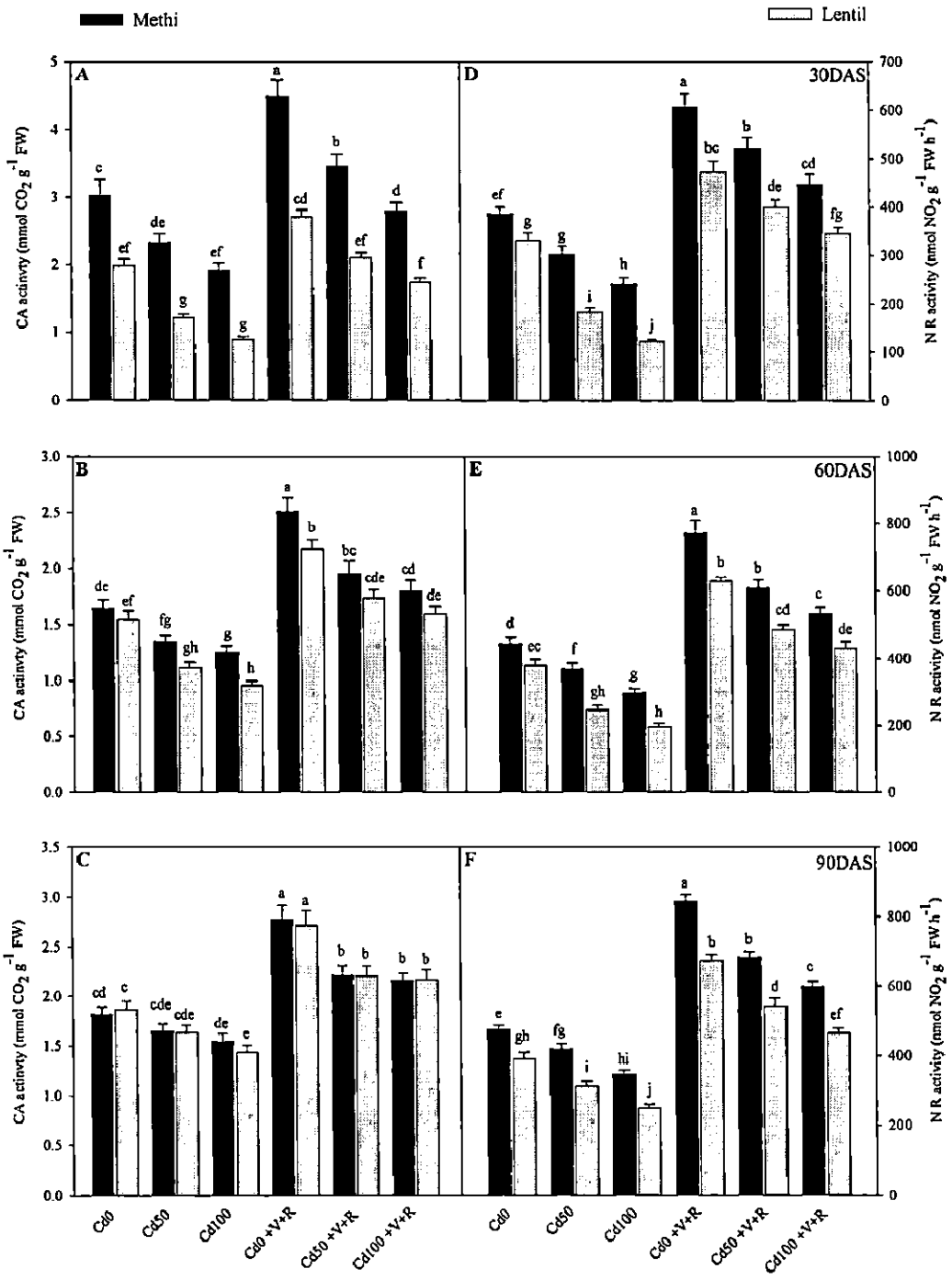


Figure 4.61: Effect of *Rhizobium* (R) inoculation and Arbuscular Mycorhizal (AM) fungi application against 0, 50 and 100 mg Cd Kg^{-1} soil on CA activity (mmol CO_2 g^{-1} FW) weight and NR activity (nmol NO_2 g^{-1} FW h^{-1}) of methi (*Trigonella foenum-graecum*) and lentil (*Lens culanaris*) at 30, 60 and 90 days after sowing.

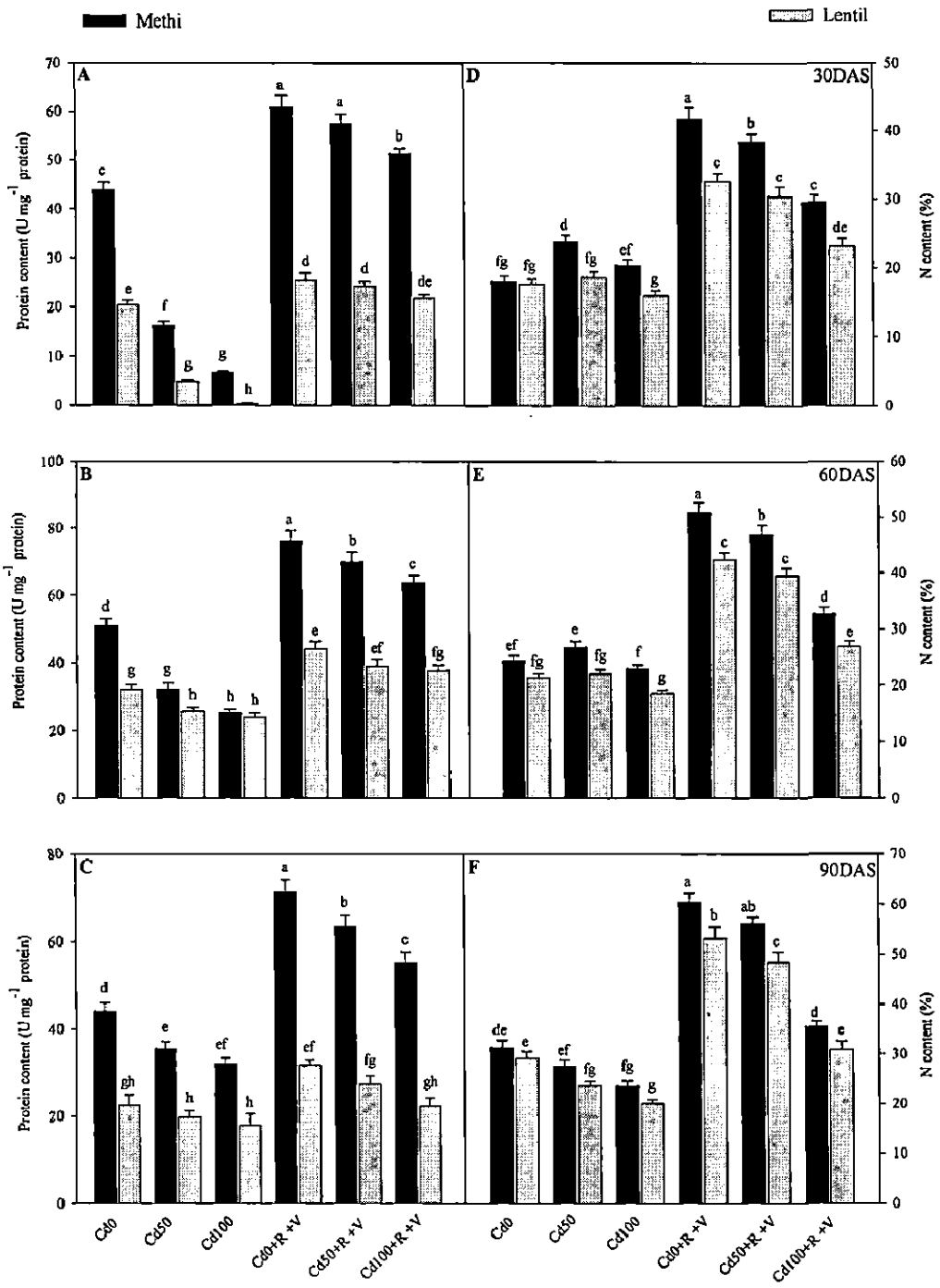


Figure 4.62: Effect of *Rhizobium* (R) inoculation and Arbuscular Mycorhizal (AM) fungi application against 0, 50 and100 mg Cd Kg^{-1} soil on protein content (U mg^{-1} protein) weight and nitrogen content (%) of methi (*Trigonella foenum-graecum*) and lentil (*Lens culanaris*) at 30, 60 and 90 days after sowing.

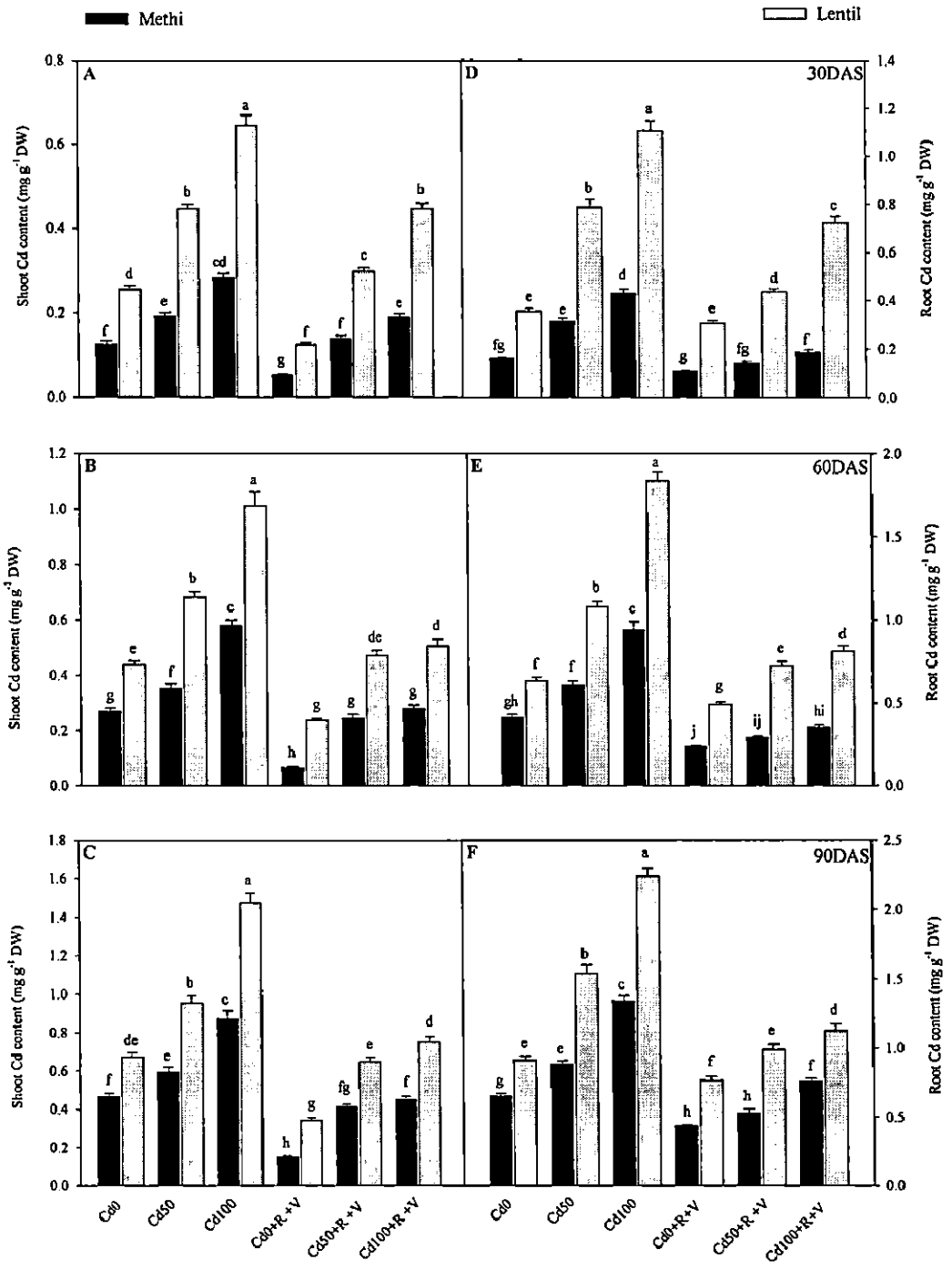


Figure 4.63: Effect of *Rhizobium* (R) inoculation and Arbuscular Mycorhizal (AM) fungi application against 0, 50 and 100 mg Cd Kg^{-1} soil on shoot Cd (µg g^{-1} DW) content and root Cd (µg g^{-1} DW) of methi (*Trigonella foenum-graecum*) and lentil (*Lens culanaris*) at 30, 60 and 90 days after sowing.

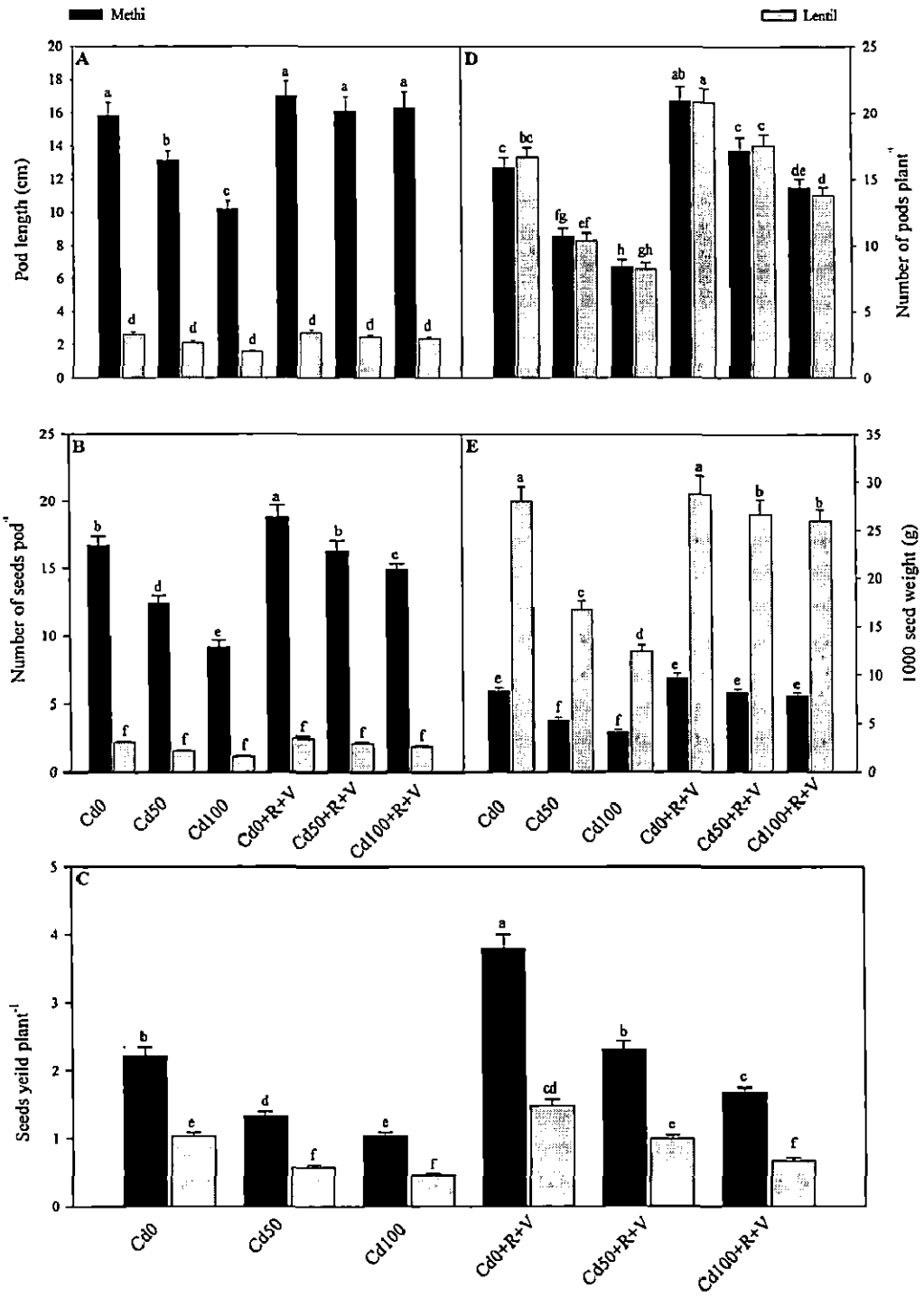


Figure 4.64: Effect of *Rhizobium* (R) inoculation and Arbuscular Mycorhizal (AM) fungi application against 0, 50and100 mg $CdCl_2$ Kg^{-1} on pod length (cm), number of pods $plant^{-1}$, number of seeds pod^{-1}, 1000 seeds weight and seed yield $plant^{-1}$ of methi (*Trigonella foenum-graecum*) and lentil (*Lens culanaris*) at harvest i.e., 120 days after sowing.

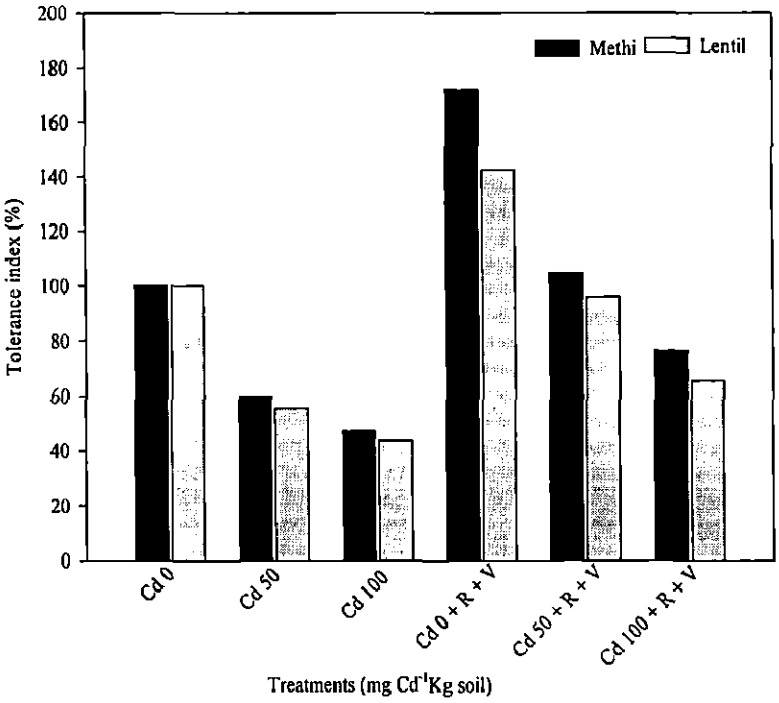


Figure 4.65: Tolerance index of two legumes (methi and lentil) exposed to 0, 50 and 100 mg Cd Kg^{-1} soil alone and seed application with *Rhizobium* and AM fungi. Tolerance index was calculated as per cent change of seed yields with control.

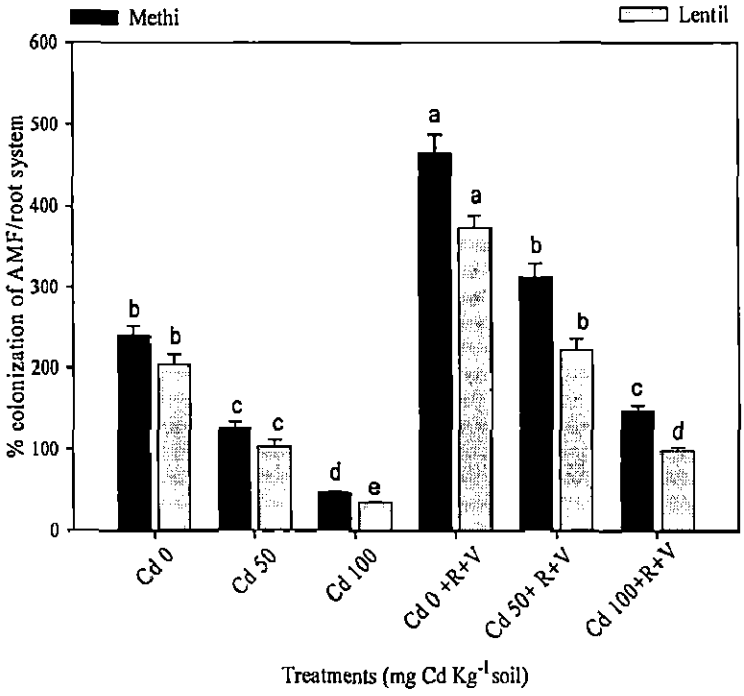
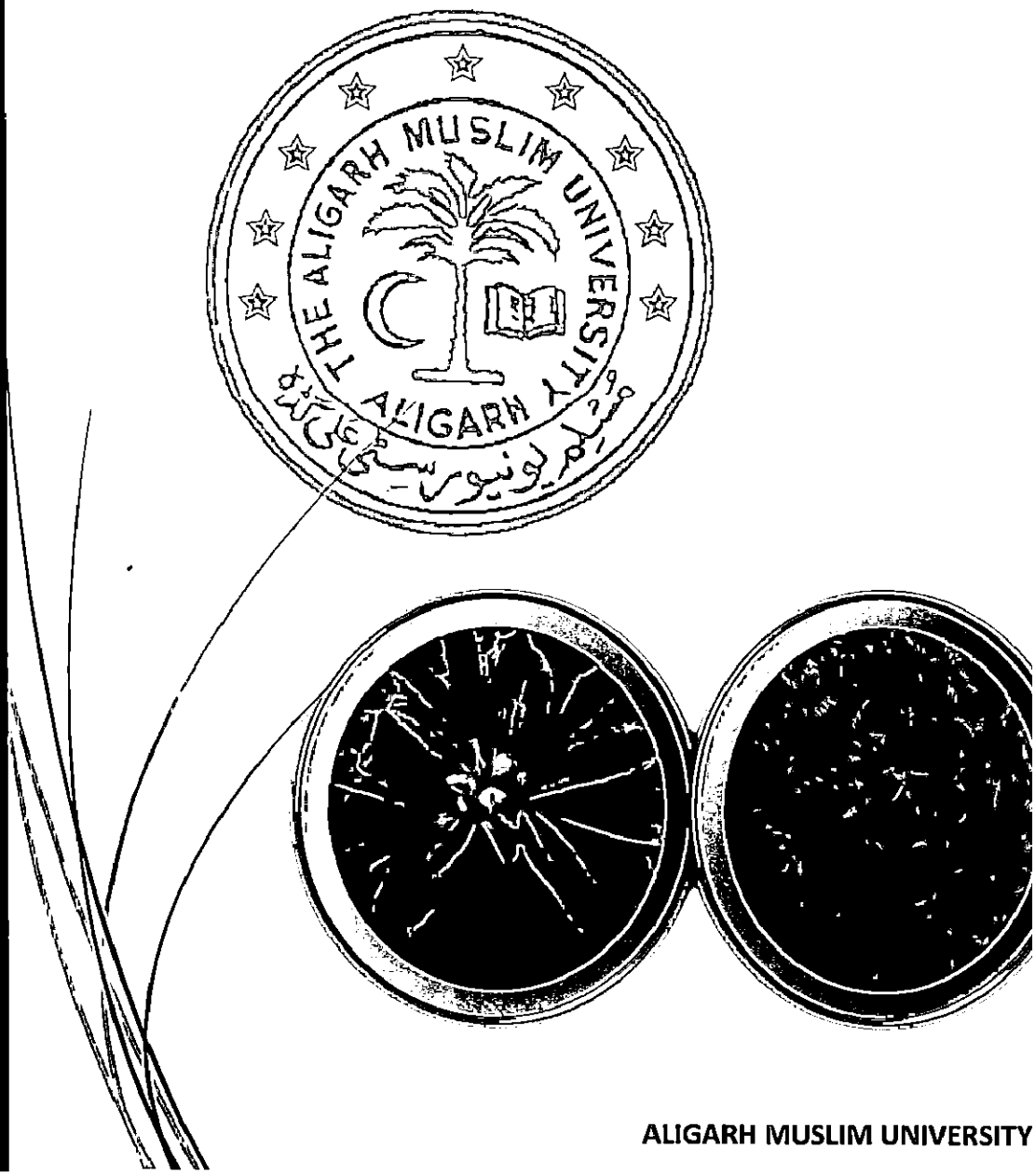


Figure 4.66: Per cent (AM) colonization in roots of two legumes (methi and lentil) exposed to 0, 50 and 100 mg Cd Kg^{-1} soil alone and seed inoculated with *Rhizobium* and AM fungi at 60 days after sowing.

Chapter-5

Discussion



CONTENTS

DISCUSSION

Heavy metal (HM) contamination of biosphere is a matter of great concern with growing urbanization, industrialization, mining and application of phosphate fertilizers in agriculture. It imposed the natural selection of certain crop plants to accumulate toxic level of heavy metals (HMs) or to negatively affect their growth and yield output. Legumes are important source of nitrogen (N) rich vegetarian diet, harboring the symbiotic N_2 fixing bacterial strains of *Rhizobium*, in their root nodules. Legumes are sensitive crops to soil HM contamination at lower threshold level, disrupting this important root-*Rhizobium* symbiosis. Although rhizobial association regulates the Cd accumulation in plant roots, the association of Arbuscular Mycorrhizal (AM) fungi, further, strengthens their N fixing capability (Garg and Bhandari, 2012), by sieving the toxic-level of accumulation of HMs. Therefore, symbiotic microbial ecosystems in the rhizosphere are potential regulators of legumes growth under inevitable HM stress conditions. The toxicity of legumes to HMs and their response to these PGPMs, though, depends upon species (genotype) and level of toxicity of HM, metal species is also an important factor to determine the response of a particular crop against the inoculated PGPM(s). Stage specific responses could be marked, culminate into reduced yield and quality of produce. Among these HMs, cadmium (Cd) takes the important place. Cadmium is a non-essential, readily accessible and phytotoxic HM with its high enrichment ratio in plants.

Concerning these above facts, five leguminous plants were taken into account to screen primarily against the increasing dose of Cd (salt of $CdCl_2$; 0, 25, 50, 75 or 100 mg Kg^{-1} soil). The least sensitive and most sensitive species were selected to test their compatibility and level alleviation of Cd toxicity due to the application *Rhizobium* and/or AM fungi.

EXPERIMENT 1 :

5.1 Screening of leguminous plants treated with different levels of cadmium

Five leguminous plants i.e. methi, broad bean, chick pea, pea and lentil were tested against different doses of Cd as compared to control plants ($CdCl_2$; 0 mg Kg^{-1} soil). The important legume plants were screened to determine their toxicity based on the level of Cd accumulation in plants, its effects on biochemical, yield attributes and stress markers. Tolerance index was also calculated on the basis of yield.

5.1.1 Cadmium accumulation in plant

The level of Cd increased in legumes in Cd dose dependent manner and also on the basis of genotype (Figure 4.11) which indicates its positive correlation with plant tissue Cd level (Figure 5.1). Cadmium has highest enrichment ratio among all HMs, (Irfan et al., 2013). The Cd accumulations in different plants depend upon multiple factors. It is a divalent cations and is taken up by the plants from the soil (Kabata-Pendias and Pendias, 1992) by using the transporters of essential metals ions (Lachman et al., 2004). Exudation of organic substances to detoxify metal in soil (Mariano et al., 2005; Yang et al., 2013); efflux of metal and binding to cell wall matrix (Kang et al., 2007) or its retention in the roots or metabolically less active/older parts (Barcelo and Poschenrieder, 1990, Rauser and Meuwly, 1995, Lux Akhtar, 2012). Secondarily, transporter proteins facilitate the efflux of toxic metal ions from the cytosol or they allow metal sequestration into intracellular compartments e.g. vacuoles (Hall, 2002) or metal is complexed with organic acids (Dalhaize and Ryan, 1995) or low molecular weight polypeptides (Kotrba et al., 1999) such as metallothionines (Killi et al., 1991), phytochelatins (Cobbett et al. 2000) etc. As legumes are HM non-accumulators (Wang et al., 2002), they adopt the strategy to avoid these metals by redirecting the root growth and root hair induction (Potters et al., 2007; Remans et al., 2012). The different levels of Cd accumulation in legume species could be due to the involvement of either of resistance mechanisms at different levels or avoidance at the level of roots. However, different legume species accumulate different levels of Cd in their tissues. The order of Cd accumulation was lentil> pea> chickpea> broadbean> methi. Such differences were also reported earlier by Santa di toppi and Gabbrielli, (1999); Remans et al., (2012) and Akhtar, (2012) in different crop plants.

5.1.2 Total chlorophyll content

Total chlorophyll content decreased with the increasing doses of Cd in soil in all the legumes however, per cent decline in this parameter was more in lentil than methi (Figure 4.7). Though, the level of accumulation was different in different species. This may be inferred to be due to the replacement of central metal ion in chlorophyll by Cd (Kupper et al., 1996; Jain et al., 2007) and also accumulation of Cd in tissue interfere the processes of biosynthesis of chlorophyll in different plants (Feng et al., 2010) and also in legumes (Bibi and Hussain, 2005; Wani et al., 2007b, c; Ahmad et

Khan et al., 2008a). Excess Cd also degrades various enzymes which are needed to synthesize the chlorophyll (Abdel Basset et al., 1995). Lesser pigment content in lentil than methi may be due to increased absorption of Cd from the root of lentil, its enhance translocation to shoot and finally increased availability in leaves (Figure 4.7). Therefore, high Cd accumulation in chloroplast disturbs its functions (Bi et al., 2009) and a negative correlation always exist between Cd accumulation and chlorophyll content in leaf at all the growth stages which further proves the degradation of this pigment by Cd (Figure 5.1).

5.1.3 Lipid peroxidation and proline content

The increased Cd transport leads to damage of biomembranes due to lipid peroxidation which is estimated of the malondialdehyde (MDA) content. In the present investigation, MDA content increased in tissues in Cd dose dependent manner (Figure 4.8). This might be attributed to increase in oxidative stress induced by free radicals which causes oxidation of membrane proteins such as ion channels, transporters, enzymes, low molecular weight protein regulators and phospholipids etc (Hall, 2002). More the Cd accumulation in tissues, higher would be the lipid peroxidation (Figure 5.1), which might increase thiol buffers pool in the plants (Balestrasse et al., 2003; Garg and Aggarwal, 2011). The maximum damaged was recorded in lentil which accumulated highest Cd content whereas minimum damage was observed in methi which had least Cd accumulation (as discussed above). Cadmium-induced increase in lipid peroxidation of biological membranes was also reported by Cakmak et al., 1991 and that of denaturation of membrane proteins increase membrane instability, ion leakage and activity of plasma membrane ATPases (Janicka-Russak et al., 2008).

Proline content also showed the same pattern as that of MDA and here also lentil showed more accumulation than methi (Figure 4.13) which is evident from the correlation curve (Figure 5.1) that shows higher stress of lentil. Membrane destabilization allows influx of positive ions along with metal and disturbs the osmotic potential of the cell. This damage needs to be effectively reduced by different in-situ repair mechanisms to avoid further side effects. The oxidative stress-induced membrane dysfunction also triggers the synthesis of enzymes for osmotic metabolites (Rentsch et al., 1996) and hence the tissue level of proline, glycine betaine and sugar alcohols etc show an increase of proline with increasing tissue Cd was marked in

correlation measurement (Figure 5.1). Proline is a multifunctional metabolite (Szabados and Savoure, 2010), it works as osmoprotectant under stress, scavenges reactive oxygen species (ROS), and protects membrane and enzymes functions (Shah and Dubey, 1998). Cadmium-induced increase in stress markers i.e. lipid peroxidation which is denoted by MDA content and proline level was also shown in mung bean (Yusuf et al., 2012), tomato (Hasan et al., 2009) and chickpea (Hayat et al. 2013b).

5.1.4 Nodule numbers

The nodules number per root system also showed a decline with increasing level of Cd in soil (Figure 4.4) and this also clear from its correlation curve (Figure 5.1). This may be due to Cd-induced increase in nodule senescence, disruption of nodule ultrastructure and inhibition of nitrogenase activity. A significant effect of increasing HM concentration on the nodule index was also shown in white clover (Manier et al., 2009). The effect of HM on plant is also dependent on the composition of soil, nodule-rhizhobial interaction and plant genetic composition (Chaudri et al., 1993; Broos et al., 2005). Cadmium is reported to interrupt the interaction of *Rhizobium* with roots for nodulation in *Cicer arietinum* and green gram (Rana and Ahmad, 2002). It was also suggested that rhizobial strains play a key role in plant tolerance to soil metal contamination (Pereira et al., 2006).

5.1.5 Carbonic anhydrase and nitrate reductase activities

The activity of CA decrease in all the legumes in response to increasing level of applied Cd in soil (Figure 4.6) it is also clear from the correlation curve that due to high accumulation of Cd in lentil compared to methi causes the reduction in the activity of this enzymes was higher in lentil. (Figures 4.6, 5.2). The decreased CA activity could be attributed to decreased availability of Zn which is a co-factor of this enzyme (Arvind and Prasad, 2005; Irfan et al., 2013) and binding of Cd to disulfide bonds of CA (Permyakov and Kretsinger, 2011). The binding of Cd to the active sites this enzyme alters its structure and function and this activity. The Cd-mediated decrease in CA activity was reported by several other workers such as Irfan et al., (2013) in mustard, Yusuf et al., (2012) in *Vigna radiata*, Hasan et al. (2009), Hayat et al. (2012) and Yadav et al. (2013) in tomato plants and Hayat (2013b) in chickpea. In the present investigation, supplementation of Cd in the soil increased Cd content in plant tissues which in turn decreased the NR activity and this decrease was more in

lentil than methi (Figure 4.7) and also a strong negative correlation between NR activity and Cd content in soil. Our findings are in agreement with several workers such as Garg et al., (1997) in *Hydrilla verticillata* and Hayat et al., (2013a) in chickpea.

Nitrate reductase is one of the the primary enzymes of N metabolism in plant which limits the growth and development (Solomonson and Barber, 1990). Cadmium toxicity on NR activity might be through its binding to the sulfhydryl group of this enzyme and thus alters its structure which finally inhibits its activity (Van Assche and Clijsters, 1990; Arun et al., 2005; Brahima et al., 2010). Decreased cellular pool of NR, mRNA and availability of Mo, the co-factor to NR (Mendel and Hansch 2002; Eckardt, 2005) could also be other contributory factors for compromised leaf NR activity in the selected legumes (Figure 4.7).

5.1.6 Leaf protein

Cadmium-induced decrease in protein synthesis is evident with the decrease of protein content in the leaves of legumes (Figure 4.10). The decreased protein content was also reported by Krantev et al., (2008) in leaves. A negative and strong correlation exists between leaf protein and different doses of Cd in soil in legumes (Figure 5.2) which may be due to the interaction of multiple factors as already discussed (Figure 5.1) and decreased nodulation adversely affects the metabolic functions of cell such as photosynthesis and protein synthesis (Balestrasse et al., 2004; Noriega et al., 2007) leading to decreased level of carbohydrates and protein content respectively. Binding of Cd to active sites of enzymes and proteins has negative consequences on their functions. High Cd content in tissues interfere with the activity of functional proteins and enzymes needed for the synthesis of CA and NR.

5.1.7 Growth characteristics

The increasing application of mg Kg^{-1} soil and plant Cd concentration progressively inhibited the growth of all the legume plants taken for the present investigation. Growth which is measured in terms of plant length, fresh mass, dry mass and number of nodules per root system decreased with the increasing in the level of Cd in soil (Figures 4.1 - 4.6) and decline in all these attributes was more in lentil than methi. Strong and negative correlation is evident between plant dry weight, number of nodules per plant and increasing concentration of Cd in soil (Figures 5.1 - 5.2).

Cadmium-induced reduction in growth attributes was also observed in *Glycine max* (Dewdy and Ham, 1997), *Pisum sativum* (Sandalio et al., 2001), *Corchorus olitorius* (Mazen, 2004), *Medicago sativa* (Drazic et al., 2006), and *Cicer arietinum* (Hasan et al., 2008). Cadmium interferes with the activity of soil microorganisms (Remans et al., 2012), induces nodule senescence (Garg and Bhandari, 2011) and decreases N_2 fixation efficiency (Chugh et al., 1992). Free Cd in metabolically active cells interfere growth metabolism, causes oxidative stress and alters the membrane permeability which causes decrease in fresh mass as shown by Wahid et al., (2008) in mungbean, Drazic et al., (2006) in *Medicago sativa* and Ekmekci et al., (2008) in *Zea mays*. The lower level of Cd decreased the weight of root and shoot in *Vigna ambacensis* (Al-Yemeni, 2001) and *Triticum aestivium* (Milone et al., 2003). Cadmium negatively influenced plant water relations, stomatal conductance in expanding bean leaves (Poschenrieder et al., 1989, Vassilev et al., 1998) which could be due to membrane peroxidation and destabilization which is shown by its strong positive correlation with MDA level (Figure 5.1). In soil, it competes with the uptake of essential mineral nutrients (Hernandez et al., 1996; Goncalves et al., 2009) causes inactivation of primary metabolic enzymes needed for sugar metabolism (Sandalio et al., 2001; Gouia et al., 2003; Doltani et al., 2006; Nada et al., 2007). It is therefore concluded that Cd accumulation in tissues on one hand, perturbs the pigment content activity of CA which decreases of photosynthetic and ultimately causes decrease in the content of photosynthates while on the other hand, it disturbed nodulation and NR enzymes which decreases protein content. The cumulative effect of feedback of all these factors lead to decrease in growth attributes.

5.1.8 Yield characteristics

Yield characteristics also decreased in all the plants with the increasing doses of Cd in soil. The negative effect of this important parameter is more effective in lentil than methi which may be due to more accumulation of Cd in tissue of lentil than methi (Figure 4.12). A strong and negative correlation exists between seed yield of legumes and increasing doses of Cd in soil (Figure 5.2). Soil HM contamination above the permissible limit results into decline in protein content in grains and number of fruits (Salgare and Acharekar 1992; Hasan et al., 2008) and this results into decreased agricultural yield (Faizan et al., 2012; Akinola and Ekiyoyo, 2006). Cadmium induced alteration in photosynthetic pigments and net photosynthetic efficiency affect source-

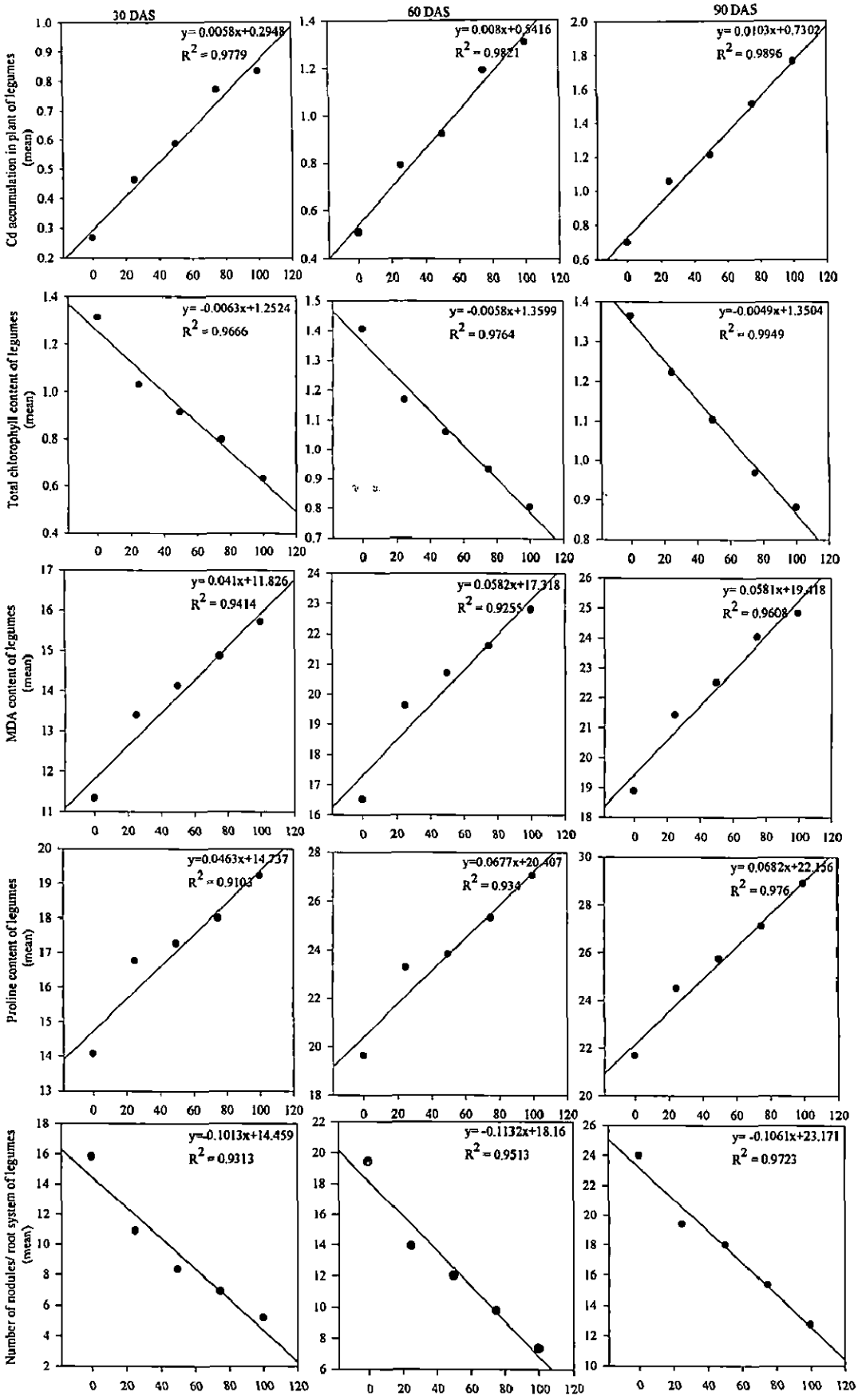


Figure 5.1: Correlation between increasing soils cadmium chloride ($CdCl_2$; 0, 25, 50, 75 or 100 mg Kg^{-1}) of five legume crops (mean values) with Cd accumulation in legumes, biochemical (total chlorophyll content, malondialdehyde and proline content of legumes) and growth (number of nodules of legumes) parameters at 30, 60 and 90 days after sowing.

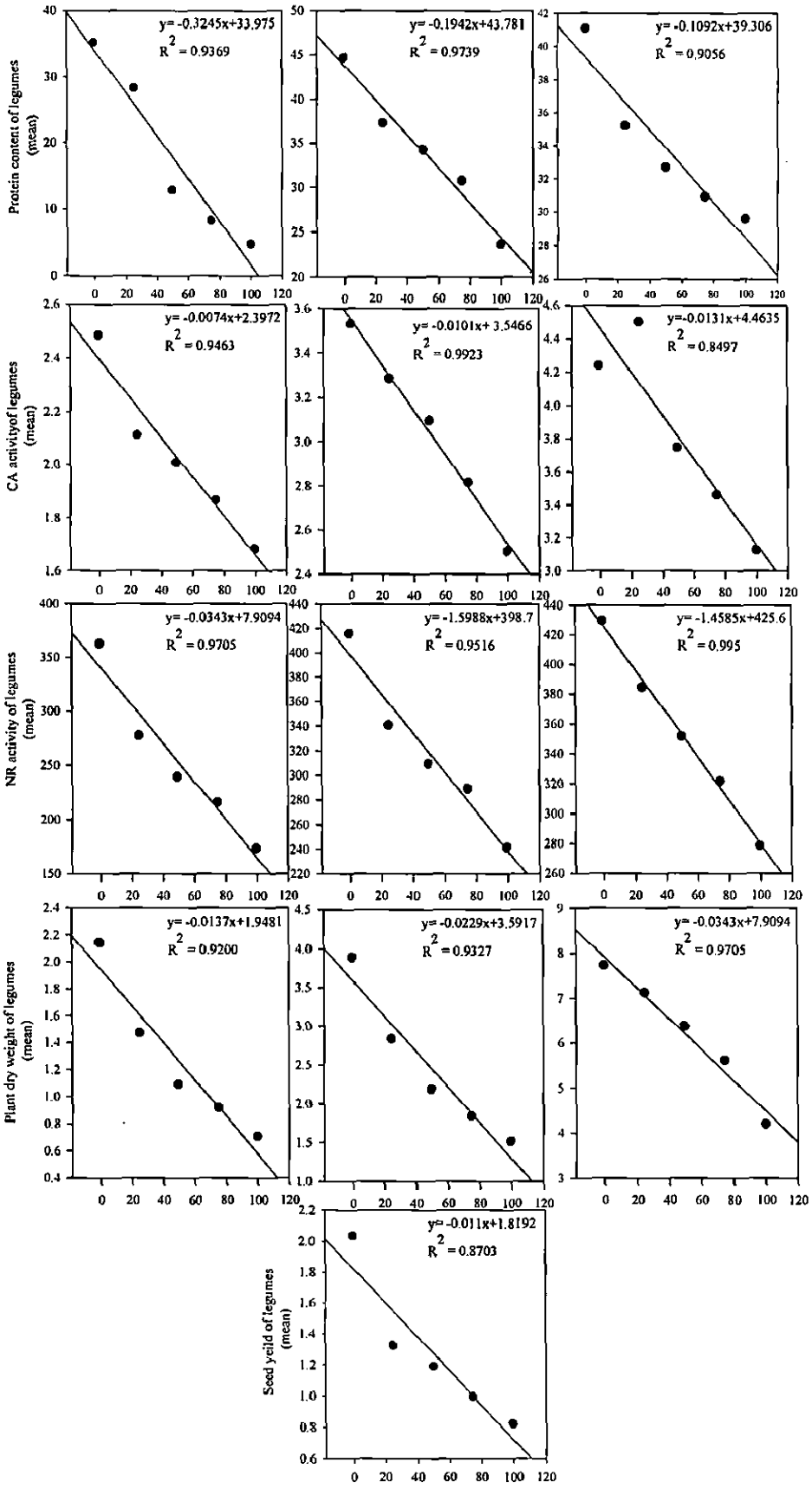


Figure 5.2: Correlation between increasing soils cadmium chloride ($CdCl_2$; 0, 25, 50, 75 or 100 mg Kg^{-1}) of five legume crops (mean values) with different biochemical (protein content, carbonic anhydrase and nitrate reductase activity of legumes) parameters, plant dry weight and seeds yield of legumes at 30, 60 and 90 days after sowing.

sink relationship and show negative correlation with dry weight accumulation and yield in a genotype dependent manner (Khan et al., 2006; Cao et al., 2014). A wide spectrum of HM induced reduction in growth and crop yield also reported by Wani et al., (2007a, 2008b) and Demirevska-Keepova, (2006). The higher reduction in yield attributes due to decrease in photosynthesis in non-tolerant variety, as compared to variety with high photosynthetic rate was earlier reported by Khan et al., 2006; and Iqbal et al., 2012; Gill et al., 2010 in mustard plants. Soil mediated Cd toxicity reduced the yield responses in chickpea (Faizan et al., 2012) tomato and mustard varieties (Hasan, 2009; Bauddh and Singh, 2011; Irfan, 2014) and weed plants (Rather, 2013).

5.1.9 Toxicity Index

The five legumes show the increasing trend of plant Cd accumulation: lentil> pea> chickpea> broad bean> methi, with the increasing soil concentration of Cd (Figure 4.13). The order of toxicity (as calculated in terms of toxicity index) was in direct correlation with Cd accumulation in selected legumes (Figure 5.2). Previously, Garg and Aggarwal (2011) observed significant inhibition of plant growth under Cd and Pb toxicity. The extent of toxicity differs among different genotypes and species, type of metal and also regulation of both at the level of root and shoot. Cadmium also interferes with the uptake of other essential nutrients. It is retained at the level of root through cell wall binding, metal efflux, regulation of transporters, exudation of organic acids, metallothionines and phytosiderophores etc. Even those metals which reach to the aerial parts are further detoxified in cytosol, sequestered to vacuoles of metabolically inactive parts, cellular homeostasis and redox balance is maintained through the increased biosynthesis and activity of antioxidant system. Based on plant dry mass accumulation, metabolic responses and yield output, methi was considered as the most resistant while lentil as least resistance to soil Cd stress. Tolerance of plants to toxicity was calculated on the basis of yield and has been used for the evaluation of genotypic variations towards the HMs (Metwally et al., 2005; Khan et al., 2006).

EXPERIMENTS 2, 3 and 4:

5.2 Alleviation of Cadmium-toxicity by *Rhizobium* and AM fungi in methi (Cd least-sensitive) and lentil (Cd most-sensitive) legumes

Legumes show high potential of symbiotic association with AM fungi than any other plant (Valsalakumar et al., 2007; Molla and Solaiman, 2009). Cadmium at high level reduces rhizobial and mycorrhizal population of soil and its symbiosis with legume plants (Sambandan et al., 1992; Pawlowska and Charvat, 2004). Alternatively, species of Glomeromycota could potentially ameliorate the HM toxicity in polluted soils (Jamal et al., 2002). Dual inoculation of *Rhizobium* and AM fungi has been shown to provide better adaptation under HM stress (del Val et al., 1999). Furthermore, the dependency of legumes to mycorrhization was reviewed by Muleta, (2010).

Therefore, considering the positive role of AM fungi and *Rhizobium* in legumes, the selected legumes (least sensitive – methi and most sensitive – lentil) were supplemented with moderate and highest dose of Cd and inoculated with either *Rhizobium* (Experiment 2), AM fungi (Experiment 3) singly or association of both the symbiont in the subsequent years of Experiment 1. Changes in growth, biochemical characteristics, stress markers, component of antioxidant system, Cd accumulation in root and shoot caused by microbial application was done at three growth stages viz. 30, 60 and 90 DAS and yield attributes at harvest (120 DAS).

5.2.1 Cadmium accumulation in root and shoot

Selected legumes show a significant difference in the accumulation of Cd at the level of root and shoot which being more in root than shoot (Figures 4.32, 4.47 and 4.63). Decreased accumulation of Cd in shoot can be correlated to the deployment of various avoidance mechanisms, mainly at the level of root. Heavy metals utilize the transporters of essential divalent cations which are essential for plants e.g. Ca, Cu and Zn etc. (Fotakis and Timbrell, 2006). This might lead to its translocation to the above ground aerial parts (Vassilev et al., 1998). Although, the legumes are sensitive to HM stress, as reported earlier in *Cicer aeritinum* (Wani et al., 2008b; Wani and Khan, 2010), *Lens culinaris* (Wani et al., 2006), *Allium sativum* as well as *Vicia faba* (Unyayer et al., 2006), *Pisum sativum* (Wani et al., 2008a) and *Vigna radiata* (Kumari et al., 2011). Rhizospheric symbionts have been reported to significantly reduce the HM availability to plants and immobilize them in soil (Rajkumar and Freitas, 2008).

The Cd accumulation decreased in the roots and aerial tissues of both the legumes when inoculated with *Rhizobium* (Figures 4. 32). Several *Rhizobium* strains show resistance to Cd and resistant strains accumulated higher levels of metals in their cell walls (Pereira et al., 2006, Ahemad, 2014).

Experiments 4 reflected that both the legumes accumulated lesser Cd as compared to plants inoculated with either of the microbes i.e. *Rhizobium* (Figures 4.32) or AM fungi (Figures 4.47) alone. AM fungi inhabiting the root cortex and rhizoplane inhibits the Cd transport to the aerial parts and bind it to thick extraradical mycelium (ERM) in roots (Joner et al., 2000) therefore, adsorbing and retaining it to underground parts of plants (Dong et al., 2007; Garg and Bhandari, 2014). Arbuscular mycorrhizal fungi reduce Cd toxicity by increasing the stabilization of HM in the soil and these fungi also exude organic acids in the soil which acts as chelating agents or they may bind the metal in the chitin of fungal cell wall (Leyval et al., 2002; Gaur and Adholeya, 2004; Gohre and Paszkowski, 2006; Karimi et al., 2011). Effective binding to the cell walls of AM fungi immobilizes metals in the fungal biomass (Joner et al., 2000; Garg and Chandel, 2010). The cell wall components of AM fungi such as free amino, hydroxyl, carboxyl and other groups can bind to Cd (Kapoor and Viraraghavan, 1995). The two legumes inoculated with AM fungi also reduced the Cd accumulation, far more than the *Rhizobium* inoculated plants (Fig. 4.47). The mycorrhizae mediated reduction of Cd accumulation could be due to Cd-buffering in the rhizosphere.

Microbes inoculated plants even at higher dose of Cd showed less accumulation. The higher concentration of HMs may delay, reduce (Lingua et al., 2008) or completely inhibit the germination and colonization of AM fungal spores (Khade and Adholeya, 2009). The effect of HM uptake by AM fungi is also depends on total concentration and speciation of metals, physicochemical chemical properties of soil and isolates of AM fungi and varieties used for cultivation (Biro et al., 2009).

Although, *Rhizobium* or AM fungi inoculated legumes accumulated lesser amount of root and shoot Cd but the effect of dual inoculation (*Rhizobium* and AM fungi) was more effective, as compared to single application (Figures 4.32 and 4.47). It is opinioned that AM fungi exude organic acids and amino acid to resist metal toxicity (Saraswat and Rai, 2011). The deprotonation of organic acids acidifies the rhizosphere which increases the mobility of metal ions or immobilizes and detoxifies

them through precipitation and complexation. A strong negative correlation exists between Cd accumulation in root and number of nodules per root system in plants inoculated with *Rhizobium* and/or AM fungi (Fig 5.3, 5.8 and 5.13) in soil supplemented with 50 and 100 mg Cd Kg^{-1} soil. Our correlation studies are in agreement with the findings of Janouskova et al., (2010) and Maynaud et al., (2013).

5.2.2 Nodule number and per cent colonization

Increasing level of Cd in soil decreased the nodulation frequency in dose dependent manner in the two legumes viz. lentil and methi (Figures 4.24, 4.39, 4.50, 4.55 and 4.66). Elevated HM levels limited the rhizobial growth and also symbiosis with their host legumes (Broos et al., 2005; Gopalkrishan et al., 2014), which may be due to decrease in nitrogenase activity (Younis, 2007). Significant decrease in acetylene reduction by nodules or soil free living heterotrophic N fixers in the presence of HMs has also been reported (Shvaleva et al., 2010) which causes substantial reduction in soil nutrient elements. Cadmium disrupts nodule ultrastructure induces oxidative damage to symbiosome membrane (Puppo et al., 2005; Garg and Manchanda, 2008) and nodule senescence. Production of ethylene, rise in NH_4^+ levels, activity of protease as well as glutamate dehydrogenase, decline in bacteroid number or symbiosome and the nodule N fixation area are the parameters related to senescence (Balestrasse et al., 2004) which results into reduced number of nodules and N assimilation (Becana et al., 2000; Matamoros et al., 2003; Garg and Bhandari, 2012; Garg and Kaur, 2012). Cadmium alters the structure of cortex, nodules and significant decrease in the content of legheamoglobin (Lb), malate, succinate and soluble proteins in the nodules of lupins (Carpena et al., 2003).

Inoculation of legumes seeds with *Rhizobium* decreased the Cd content in shoot and root tissues and this decrease was more pronounced at lower concentrations (Figure 4.24). Lower dose of Cd along with inoculation of *Rhizobium* gave more pronounced chances of Cd immobilization in soil and it's binding to bacterial cell wall. Bacteria also secrete organic acids which act as chelators to detoxify HMs in soil to reduce their uptake in plants. Further, a higher population of rhizobia in the soil has better chances of resistant strain selection under Cd stress (Kumar, et al., 2003; Pereira et al., 2006). The rhizobia which are resistant to Cd could induce better nodulation in roots of legumes. However, low dose of Cd with *Rhizobium* has also been reported to have positive effect on plant growth responses (Jia et al., 2012).

The AM inoculated legumes also show increased nodulation (Figure 4.39) which might be due to their synergistic interaction with the natural population of rhizobia inhabiting the soil under toxic Cd stress environment and thus, enable rhizobia to interact with root hairs and induce nodulation. Reduction in nodulation, nitrogenase activity and length of plant size was overcome by dual inoculation in white clovers grown in soil contaminated with HMs (Nishita and Joshi, 2010). This could be due to the resistance of bacteria in the natural population which restricts the uptake of Cd when inoculated with AM fungi (de Andrade et al., 2005; Wani et al., 2008a). Dual inoculation of symbionts could further enrich nutritional status of plant (Barea et al., 2002; Abusuwar and Ahmad, 1997, 2003; Allen and Shachar-Hill, 2009) to support its vigor (Nidhi and Rahangdale, 1999; Jia et al., 2004; Zaidi and Khan 2007; Arumugam et al., 2003, 2010).

However, the dual inoculation gave a synergistic response to nodulation (Figure 4.55) which may be due to availability of nutrients and decrease in Cd toxicity. Vast of literature available on this aspect shows that inoculations of AM fungi encourage nodulation and N_2 fixation in legumes (Bethlenfalvay et al., 1982; Champavat, 1990). Arbuscular mycorrhizal fungi facilitate the absorption of phosphorous (P) from root which is required for nodulation and N_2 fixation (Barea and Azcon-Aguilar, 1983). The increased response of nodulation efficiency by coinoculation of rhizobia and AM fungi (Toro et al., 1998). Faizan, (2002) reported improvement in protein content, NR and nodulation due to dual inoculation in lentil and chick pea in fly ash amended soil. It is also clear from the correlation study (Fig 5.6, 5.11 and 5.16) which shows a strong positive correlation between number of nodules per root system and shoot dry weight in plants inoculated with *Rhizobium* and/or AM fungi grown in soil supplemented with 50 and 100 mg Cd Kg^{-1} soil.

5.2.3 Leaf N, P, and K content

Nitrogen is an essential macronutrient which is required for plant growth and metabolism (Scheible et al., 2004) and it required for the synthesis of proteins, enzymes, chlorophyll and nucleotide. Cadmium toxicity disturbs the N metabolism of crop plants (Wang et al., 2008; Du et al., 2009). Even lower concentration of Cd could be toxic to microbial symbiosis (Pereira et al., 2006; Younis, 2007). It affects nodulation and N fixation in soybean and lupin (Chugh et al., 1992; Chen et al., 2003; Balestrasse et al., 2005, 2006; Younis, 2007). In the present investigation, decrease of

N content due to Cd stress may be due to decrease in the activity of *Rhizobium* in nodules to fix N, limited nitrate absorption and activity of nitrate reductase. Soil Cd-mediated decrease of N content was reported in the *Avena sativa* grain, *Lupinus luteus*, *Phacelia tanacaetifolia* and of *Zea mays* (Ciecko et al., 2001, 2004). In my study, resistant inoculum of *Rhizobium* has partially overcome Cd-mediated decrease in macronutrient acquisition in plants and its inoculation to seeds enhanced the N level in lentil and methi (Figure 4.31).

As discussed above, Cd might be detoxified by the cell wall components of this bacteria, and the activity of bacteria might have enabled the legumes to form and protect nodules and also helps in the uptake of fixed N to the above ground part. Furthermore, PGPRs have also been reported which produce phytohormones in the soil which could further ameliorate the Cd-mediated toxicity at the level of root (Wani et al., 2008b; 2009). Chaer et al., (2011) and Hao et al., (2014) observed legume-rhizobia symbiosis can restore biological N_2 fixation and can increase the accumulation of N and organic matter in HM contaminated soils.

Inoculation of AM fungi, improves the N status of the two legumes (Figure 4.46). The intra radical mycelia (IRM) found within the root is the site of nutrient exchange between the symbionts, while the extra radical mycelia (ERM) extends beyond the depletion zone and increase the available surface area for absorption of nutrients specially N which can be taken up in the form of ammonium, nitrate from organic sources (Allen et al., 2009; Leigh et al., 2009). Studies indicate mycorrhizae to assist N and P uptake of plants (George et al., 1995; Hawkins et al., 2000) helps plants to colonizing in metal contaminated sites which resulted into enhanced growth and yield output (Rivera-Becerril et al., 2002, Vogel-Mikus et al., 2005; Lin et al., 2007).

The dual inoculation of AM fungi and rhizobia improves N and P in plants compared to either of their single inoculation (Barea et al., 2002; Jia et al., 2004). Applications of both the symbiont caused a substantial gain of availability of other nutrients (Allen and Shachar-Hill, 2009; Ndoye et al., 2014) and promote plant growth and yield either under stress free and stress full conditions in several other plants (Bhattacharjee and Sharma, 2012; Patil et al., 2013; Ashrafi et al., 2014). In the present study, supplementation of Cd to soil decreased the P content of selected legumes (Figures 4.24, 4.39 and 4.55). This decrease was attributed to be due to

decreased activity of microbes in the soil which results in decreased uptake of phosphate ions (Jing et al., 2007, Saharan and Nehra, 2011, Vacheron et al., 2013).

Rhizobium inoculation has been shown to increase the N and P level in legumes as compared to non-inoculated plants (Hussain et al., 2012, Murtaza et al., 2014). Increased availability of P with *Rhizobium* inoculation positively regulates the nodulation as in present experiment (Figure 4.24 and 4.31), mungbean (Hussain et al., 2012) and *Vigna mungo* (Murtaza et al., 2014).

Arbuscular mycorrhizal symbiosis increase P uptake which is required for nodulation and N_2 fixation processes (Lukiwatid and Simanungkalit, 2002; Jia et al., 2004). The role of AM fungi in the acquisition of mineral nutrients, especially P was explained previously by some scientists (Pacovsky, 1986; Naushin, 1998 and Jackobson, 1999). Smith et al., (1986) demonstrated the increase in growth of *Allium cepa* when inoculated with AM fungi. Increased uptake of P from soil to plant due to AM inoculation has been studied in *Vigna radiata* (Sekhon et al., 1992) and pea plants (Geneva et al., 2006). This fungus improves P acquisition even in infertile soil also (Hu et al., 2009; Boddington and Dodd, 2000; Zaidi et al., 2003). Mycorrhizal mycelia provide a larger surface area for the absorption of water and minerals and they can also explore a greater volume of soil. Phosphorous has been reported to be critical element in the formation of nodules (Toro et al., 1998; Malekzadeh et al., 2007).

Legumes inoculated with *Rhizobium* and AM fungi increased the N and P assimilation (Geneva et al., 2006) and hence the nodule and plant biomass also (Nidhi and Rahangdale, 1999). Arbuscular mycorrhizal fungi when used either alone or in combination with other rhizosphere microbes can enhanced plant growth including legumes both in conventional (Zaidi et al., 2003; Zaidi and Khan, 2006) and in HM-contaminated sites, by increasing plant access to relatively immobile minerals such as P (Yao et al., 2003) and K (Chen et al., 2007). AM fungi increased P content under Cd stress in tomato.

Cadmium mediated decrease of potassium (K) level in leaves, has been observed in the selected legumes (Figures 4.28, 4.43 and 4.59). This decrease appears due to membrane damage and leakage of ions. The uptake of K varies depending upon species and organ, under Cd contamination. Potassium content decreased in oat

grains, in the above ground parts and roots of yellow lupine and radish in soil contaminated by Cd (Ciećko et al., 2004). Saadati et al., (2012) tested two commercial varieties of pinto bean (*Phaseolus vulgaris*) against different levels of soil Cd with soil amended with K. The treatments significantly increased the uptake of plants K level, which resulted into increased proline level and enhanced resistance of these varieties to Cd. In the leaves of *Vicia faba*, Ca^{2+} and K^{+} ions has been shown to improve the antioxidant enzymes activity along with growth (Hayat et al., 2011) and yield (Ciećko et al., 2004) under Cd toxicity. In this experiment also the lentil showed higher K level as compared to methi in all treatments.

The level of K increased in plants inoculated with *Rhizobium* (Figure 4.28). Manoharan et al., (2008) observed increase in K acquisition under AM symbiosis. Bethlenfalvay et al., (1982) reported that plant and nodule dry weight increased under low P availability due to dual inoculation of *Rhizobium* and *Glomus fasciculatum* in *Phaseolus vulgaris*. When the availability of P is low, increased P uptake was observed in *Glycine max* (Bethlenfalvay and Yoder, 1981). This was also favored by a strong and positive correlation between leaf per cent N with protein content and number of nodules per root system with per cent P in plants inoculated with single or dual inoculation of *Rhizobium* and AM fungi (Fig 5.3, 5.4, 5.8, 5.9, 5.13 and 5.14) in soil supplemented with Cd (50 and 100 mg Kg^{-1} soil).

5.2.4 Photosynthetic pigments

The photosynthetic pigments i.e. chlorophyll (Chl a, Chl b and total Chl) and carotenoids content decreased in the leaves of lentil and methi, with the increase doses of Cd in soil (Figures 4.25, 4.26, 4.40, 4.41, 4.56 and 4.57). The decreased content of chlorophyll and carotenoids was also reported in different plants with the increasing level of HMs (Baryla et al., 2001; Verma and Dubey, 2002; Manoharan et al., 2008). Carotenoids serve as antioxidants against free radicals and photochemical damage (Sengar et al., 2008). Thus less effect on carotenoids might represent its supportive role against oxidative stress (Kelman et al., 2009). Heavy metals are reported to decrease the chlorophyll content, carotenoid/chlorophyll ratios and photosynthetic performance in algae, (De Filippis and Pallaghy, 1994; dos Santos et al., 2012) besides in higher plants e.g. in carrot, potato (Flemotomou et al., 2011) and *Vigna mungo* (Singh et al., 2008). The decreased level of chlorophyll pigments, in response to Cd accumulation in plant is primarily associated with disturbed membrane

organization of thylakoids and chloroplast ultrastructure in *Salix purpurea* and *Phragmites australis* (Hakmaoui et al., 2007). Parmar et al. (2013) reviewed the cadmium induced structural and functional changes in photosynthetic apparatus. Cadmium mediated excess generation of ROS may induce degradation of chlorophyll content (El-beltagi et al., 2010). It may induce lipoxygenase activity which contributes chlorophyll oxidation (Somashekaraiah et al., 1992). Decreased chlorophyll synthesis may partially be due to inhibition of chlorophyll biosynthetic enzymes (Noriega et al., 2007, Muneer et al., 2011) or induced synthesis of enzymes which catabolize it (Abdel Basset et al., 1995) and ultimately reduce the photosynthetic activity in leaves of soybean (Xue et al., 2014). It also competes with several essential divalent cations in the rhizosphere such as Mg, Fe and Ca etc. and affects several other physiological functions besides the activity and metabolism of chlorophyll molecule (Ouzounidou et al., 1997; Nazar et al., 2012). Chlorosis might be due to the changes in Fe:Zn ratio caused by this and the negative effects in chlorophyll metabolism (Baryla et al., 2001; Chaffei et al., 2004). Cadmium decreased chlorophyll level is also in conformity with the similar works done in wheat (Amani, 2008) and *Catharanthus roseus* (Pandey et al., 2007).

Finding of Experiment 2 reflected improvement in pigment contents in the leaves of two legumes when inoculated with *Rhizobium* (Figures 4.25 and 4.26). The induction of chlorophyll biosynthesis could be due to facilitated uptake of Mg ions, N supplementation to tetrapyrole rings of chlorophyll-head or hormonal induction. The increased pigment content in the leaves due to *Rhizobium* inoculation was earlier reported by Wani and Khan, (2012) in lentil.

Selected legumes reflected an improvement of chlorophyll contents when inoculated with AM fungi (Experiment 3).These findings are in agreement with those of Bhosale and Shinde, (2011) and de Andrade et al., (2008) who reported an increase in photosynthetic pigment contents in *Zingiber officinale* and *Helianthus annuus* respectively in the presence of Cd. The improvement in pigment content may be due to decrease in Cd accumulation and hence reduced toxicity in leaves. Different workers have shown that amount of chlorophyll was higher in mycorrhizal plants than non-mycorrhizal plants (Mathur and Viyas, 1995; Gemma et al., 1997; Abad and Khara, 2007; Malekzadeh et al., 2007). Asrar and Elhindi (2011) reported that

photosynthetic pigments were stimulated by AM symbiosis under different abiotic stresses (Colla et al., 2008; Fiazan, 2002; Kaya et al., 2009; Haji boland et al., 2010).

Co-inoculated plants shows further gain in pigments contents in Cd amended soil (Figures 4.56 - 4.57). Arumugam et al., (2010) also found a favorable response on chlorophyll content in *Vigna unguiculata* inoculated with AM fungi and *Rhizobium*. This finding is also favored by a strong and positive correlation between number of nodules per root system with total chlorophyll content and total chlorophyll content with shoot dry weight and per cent leaf P and carotenoid content when plants were inoculated with single or dual inoculation of *Rhizobium* and AM fungi (Fig 5.3, 5.4, 5.6, 5.8, 5.9, 5.10, 5.13, 5.14 and 15.16) in soil applied with Cd.

5.2.5 Carbonic anhydrase, nitrate reductase activities and leaf protein content

The enzyme CA is an important metalloenzyme required for reversible hydration of CO_2 to HCO_3, requires Zn for its activity (Xin Bin et al., 2001; Khan, 2004). The activity of CA inhibited with the increase in Cd toxicity in soil (Figures 4.30, 4.45 and 4.61). The inhibition of CA activity in plants is multifaceted. The scarce availability of substrate (CO_2), stomatal conductance, availability of Zn, light intensity, hormonal regulation and direct inhibition of CA activity (transcription and translation) could contribute to change in the activity of CA (Aravind and Prasad, 2005; Tiwari et al., 2005; Escudero-Almanza et al., 2012; Wei-Hong et al., 2014). Cadmium induces ABA and NO mediated stomatal closure, and competes for Zn in the root zone (as previously discussed). Direct inhibition of enzyme is due to binding of Cd to –SH groups of enzyme CA, Cd induce the degradation of mRNA (Park et al., 2012). Cadmium mediated decrease in the activity of this enzyme was shown by Hayat et al., (2007) and Hasan et al., (2007, 2008).

Carbonic anhydrase activity enhanced when legumes were inoculated with symbiont. However, this activity was higher in AM inoculated plants as compared to *Rhizobium* inoculated plants (Figures 4.30 and 4.45). Although, maximum improvement in the activity of this enzyme was recorded in co-inoculated plant under Cd stress in soil. AM fungi increased absorption of mineral elements and the two symbionts together reduce Cd translocation to shoots (Abdel-Latef, 2013). Decline in Cd accumulation in shoot along with increased availability of Zn, the co-factor of CA facilitates induction of the activity CA (Escudero et al., 2012). Furthermore, Cd

permeation disturbs plant water status (Perfus-Barbeoch et al., 2002) leading to normal stomatal conductance and gaseous exchange (Neill et al. 2007, Besson-Bard et al. 2009; Gill et al., (2010). Therefore, sufficient availability of substrate (CO_2) could have positive feedback of CA activity (Xin Bin et al., 2001; Khan, 2004). *Rhizobium* symbiosis due to enriched N and protein proteins, may cause positive feedback to the cellular CA pool at translational level. Reduction in Cd induced oxidative stress and phytohormonal induction may further support the cellular environment to the favor the CA transcriptional and translational machinery at it is optimum for the formation of CA.

Nitrate reductase is a key enzyme of N assimilation in plant (Sivasankar et al., 1996) which catalyzes the conversion of nitrite to nitrate (Larcher, 1995). The NR activity in the leaves of two legumes concomitantly decreased with the increase of Cd doses. Cadmium also acts as an inhibitor of NR enzyme (Keshan and Mukherjee, 1994). Cadmium mediated change in plasma membrane fluidity can induce the partial loss of NR activity (Meharg, 1993) thus alter proton pump of the membrane (Obata et al., 1996) and N uptake (Campbell, 1999; Wani et al., 2007). The absorption of nitrate is an energy dependent active process. A reduced supply of NADP and H ATP is suggested to disorganize the chloroplast (Sharma and Dhiman, 2013) due to change in cytoplasmic energy pool; Cd disrupts organization of membrane or blocks the nitrate transport system which further restricts the nitrate absorption (Hernandez et al., 1997; Obata et al., 1996). Moreover, the reduction of nitrate absorption at the root level may be due to Cd mediated water loss from root cell membrane and transpiration inhibition (Hernandez et al., 1996). The assimilation of N is hindered by the Cd disrupted cellular activities which retards biosynthetic activities and interrupts action of the specific enzymes (Dube et al., 2009). Its toxicity affects the enzymes of N metabolism (Chugh et al., 1992; Singh et al., 1994; Chaffei et al., 2004). Cadmium induces a significant decrease in nitrate content, inhibits the activities of nitrate reductase and nitrite reductase in tomato (Chaffei et al., 2004), *Phaseolus mungo* (Siddhu and Khan, 2012), *Arachis hypogea* (Dinakar et al., 2009) and beans (Gouia et al., 2000).

Selvaraj, (1998) also reported increased NR activity due to inoculation of AM fungi. Arbuscular mycorrhizal fungi are known to assimilate and transport both NH_4^+

ions and some organic N compounds to their host plants, particularly under condition of low N availability and low pH.

Toxicity of high concentration of HM inactivates protein synthesis (Figures 4.31, 4.46 and 4.62) this is in agreement with the finding of Van Assche and Clijsters, 1990; Brahima et al., 2010. Cadmium induces oxidative stress, which oxidizes low molecular weight regulatory biomolecules such as proteins, translation proteins, kinases, phosphatases and regulatory RNAs etc. The protein biosynthesis and cell-cycle progression checked. Cadmium stress may activate ubiquitin-mediated protein degradation to induce programmed cell death.

Arines et al., (1993) reported increased protein content in red clover inoculated with AM fungi. Plants amended with Cd and inoculated with AM fungi increased the level of proteins in wheat (Abad and Khara, 2007) tomato (Malekzadeh et al., 2007) and *Capsicum annum* (Abdel Latef, 2013).

Andrade and da Silveira (2008) observed that *Glomus macrocarpum* promoted plant growth, increased protein contents and alleviated the nutritional stress caused by Cd stress in plants inoculated with mycorrhizae in *Zea mays*. *Glomus mosseae* treatment decreased soluble proteins in *Vicia faba* grown in soils contaminated with Cd, Pb, Cu and Zn (Zhang et al., 2006). The molecular proteomic responses of AM plants indicate altered expressions of several proteins under Cd stress (Alouie et al., 2009). Finlay (2008) suggested that AM improves protection against toxic metal-induced oxidative stress through strongly induces glutathione synthesis. Glutathione S-transferases (GST) catalyze the conjugation of glutathione with various reactive electrophilic compounds and may provide protection against oxidative stress (Saraswat and Rai, 2011).

However, dilution of free cellular Cd might decrease these consequences to recover cellular machinery of protein synthesis. Plants inoculated with AM fungi show increased protein synthesis in vegetable seedlings (Lenin et al., 2012; Manila and Nelson, 2014), tree seedlings (Manoharan et al., 2008) and legume plants (Bhattacharjee and Sharma, 2012), which indicate retention of Cd at the level of root and protection of *Rhizobium* symbiosis, increased the availability of N for protein synthesis. A strong and positive correlation between number of nodules per root system with leaf NR activity, and chlorophyll content with leaf CA activity in plants

inoculated with *Rhizobium* and/or AM fungi (Fig 5.4, 5.9, and 5.14) in Cd supplemented soil (50 and 100 mg Cd Kg^{-1}) further favors above statement.

5.2.6 Lipid peroxidation and proline content

Cadmium-induced toxicity symptoms attributed to oxidative damages arising from imbalance in the generation and removal of ROS (Molina et al., 2008; Zheng et al., 2010; Anjum et al., 2011). Overproduction of H_2O_2 leads to increased leakage of ions from roots or leaves in different plant species exposed to different Cd concentrations (Hatata and Abdel-Aal, 2008; Anjum et al., 2011) potentially due to induction of NADP oxidases and superoxide dismutases (Heyno et al., 2008; Olmos et al., 2003). Cadmium exposure causes oxidative injuries, such as lipid peroxidation which leads to alteration in the membranes function, fatty acid composition and protein carbonylation (Popova et al., 2009; Dalcorso et al., 2008) which can be determined by estimating the content of MDA (Tamas et al., 2009; Zhiqiang et al. 2009; Singh et al., 2010). In the leaves of two selected legumes, soil Cd treatment sharply induced the MDA level (Figures 4.27, 4.42 and 4.58) indicating membrane lipid oxidation and damage. Cadmium-induced lipid peroxidation has been widely observed in different crops and wild plants (Dominguez et al., 2010; Yang et al., 2011). Cadmium also disturbs the membrane stability alter the selectivity of protein channels and transporters, which determines the tolerant and susceptible features of cultivars. Some crops with higher membrane stability can be correlated with Cd stress tolerance (Mobin and Khan, 2007). In the present investigation, the MDA content decreased with the inoculation of *Rhizobium* partially because detoxification of Cd at the root level, increased Cd resistance and due to enriched N supplementation to strengthen plant defense.

Alternatively, AM fungi inoculation further decreased MDA content under Cd stress in both the legumes (Experiment 3). Significant reduction in lipid peroxidation with AM inoculation is in conformity with those Garg and Aggarwal, (2012) on pigeon pea who found significant decrease of TBARS content in leaves and roots of AM plants than in the non-AM plants under both metal treatments. Wu and Zou, (2009) also reported significant lower content of MDA in mycorrhizal plants *Citrus sinensis* (*Trifoliata orange*) throughout the drought stress period. Similarly, Rahmaty and Khara, (2008) found decline in the content of MDA in the root and shoot of maize plants inoculated with AM fungi than non-AM Plants in grown under Cr stress. The

result was similar to that obtained in *Arabidopsis thaliana* plants (Maksymiec et al., 2007) and in *Helianthus annuus* leaf disc (Groppa et al., 2001). In the present study (Experiment 4) inoculation of AM fungi and *Rhizobium* maximally and significantly lowered the MDA content against both the concentrations of Cd (50 or 100 mg Kg^{-1} of soil) treated plants, and enhanced the effectiveness of *Rhizobium* association.

Both the plants exposed to Cd showed rapid accumulation of proline. At higher concentration, proline accumulation was further enhanced. However, lentil showed more accumulation compared to methi. Kasai et al., (1998) reported that increase in proline content may be either due to de novo synthesis or decreased degradation of proline or both the reasons. Our results are in agreement with several workers who obtained similar results on bean shoots (Bavi et al., 2006) *Helianthus annus* L. seedlings (Zengin and Munzuroglu, 2006), *Triticum aestivum* (Abdel-Latif, 2008), *Solanum. melongena* L. cv Hybrid PK123 (Dube et al., 2009), *Vigna radiata* (Muneer et al., 2011) and *Cicer arietinum* (Faizan et al., 2011). Cadmium induces membrane (lipid and protein) oxidation and brings about change in membrane functionality and permeability (Nazar et al., 2012). Plants manage this perturbed cellular homeostasis by synthesizing low molecular weight compatible solutes (Sharma and Dietz, 2006) such as proline, total soluble sugars, glycine betaine, trehalose, sorbitol etc. These osmolites act as osmoprotectants, cellular protein stabilizers (Sharma and Dubey, 2004; Sharma and Dietz, 2006) inhibitors of lipid peroxidation and free radical scavengers (Alia et al., 2001). Proline is a multifunctional metabolic stress indicator (Van Heerdena and de Villiersa, 1996; Szabados and Savoure, 2010). It feeds on excess ROS, hydrates functional proteins and enzymes of cytoplasm and membrane, and maintains the cellular osmolarity (Hayat et al., 2012) to increase the performance of enzyme proteins of antioxidant system and photosynthetic machinery (Hayat et al., 2013b). Also, in the Experiment 2, 3 and 4, the level of proline progressively increased with the increasing soil (and hence shoot) Cd concentration of leaves of the two legumes. Several other reports also suggests significantly increased proline accumulation with the increasing Cd concentrations, as in the shoots of bean (Bavi et al., 2006), seedlings of *Helianthus annus* L. (Zengin and Munzuroglu, 2006), *Triticum aestivum* (Abdel-Latif, 2008), *Solanum melongena* L. (Dube et al., 2009), *Vigna radiata* (Muneer et al., 2011) and chickpea (Faizan et al., 2011).

The inoculation of *Rhizobium* decreased the proline level in the leaves of two legumes (Experiment 2), possibly due to *Rhizobium* mediated decline of Cd uptake and improvement of plant defense system through Cd adsorption on cell wall, secretion of phytohormones and improved N-metabolism (Wani et al., 2008c; 2009).

However, with the inoculation of AM fungi in Cd stressed legumes, the level of leaf proline content decreased. AM fungi has been shown to increase tolerance of plant to metals through, modification of proline and polyamine metabolism for better stabilization of metal in roots and more efficient response (Paradi et al., 2003; Ruscitti et al., 2011; Abdelmoneim et al., 2014). Courtecuisse, (1999) reported that AM might cause decrease in proline accumulation which may be due to its role in mediating the uptake of water at the time of drought stress and HM contaminations. The involvement of AM fungi reducing the proline content was in conformity with the findings of Bhosale and Shinde, (2011) in *Zingiber officinale*

The co-inoculation, therefore, potentially synergized the soil detoxification and improved the plant health to improve its defense system and tolerance. From the fining of Experiment 4, it was inferred that dual inoculation the dual inoculation improved the proline level in the leaves of legumes. A strong and positive correlation exists between Cd accumulation in shoot with leaf proline content and Cd accumulation in shoot with leaf MDA level, when plants were inoculated with *Rhizobium* and/or AM fungi (Figures 5.5, 5.10, and 5.15) in soil amended with 50 and 100 mg Cd Kg^{-1} soil.

5.2.7 Enzymatic antioxidant

The activity of SOD, CAT and POD increased with the exposure to Cd at all the three doses in the two legumes whereas highest increase was recorded at early growth stages. The accumulation of H_2O_2 and O_2^- could reflect the oxidative stress and changes in antioxidant defense system in plants (Romero-Puertas et al., 2004). Plants cope with oxidative stress intricating antioxidative system which is responsible for scavenging excessively accumulated ROS in plants under stress in general (Sbartai et al., 2008; Sharma et al., 2012; Weisany et al., 2012) and metal stress in particulars (Azcon et al., 2009; Saraswat and Rai, 2011; Gill et al., 2013). The expression and activation of these enzymes viz. peroxidase (POD), superoxide dismutase (SOD) and catalase (CAT) differs as per HM toxic dosages, exposure time and species or

exposed organs of plant. The activity of these enzymes increase with the increase in free radicals, however, it may decline with excess or suboptimal level of ROS. Efficiency of enzymes may also decline due to oxidation of transcripts or proteins of these enzymes or other regulatory factors. Increase in total SOD activity was detected due to the application of Cd in several plant species (Schutzendubel et al., 2001). Cadmium stress increases the SOD activity in the roots of mung bean which decreased after 8 days, whereas, the CAT activity in the leaves of Cd-treated seedlings decreased (Molina et al., 2008). Moreover, a positive correlation between Cd stress and the abundance of SOD in different tissues of *Glycyrrhiza uralensis* plants was also observed. Like SOD, CAT activity increases in sugarcane (Fornazier et al., 2002) while in soybean (Ferreira et al., 2002) it had no effect. Cadmium increased the total POD activity in *Ceratophyllum demersum* (Aravind and Prasad, 2003) and *Arabidopsia thaliana* (Cho and Sohn, 2004).

Arbuscular mycorrhizal fungi reduce the level of lipid peroxidation due to decrease in the accumulation of Cd. Increase in the efficiency of antioxidant enzymes was observed in plants when colonized by AM fungi (Soltani et al., 2006; Abad and Khara, 2007). Also, a remarkable increase in antioxidant enzymes in *Spartina densiflora* (Dominguez et al., 2010), *Glycyrrhiza uralensis* (Zheng et al., 2010) and *Raphanus sativus* L. (El-Beltagi et al., 2010) was recorded when plants were exposed to Cd.

However, legumes inoculated with *Rhizobium* or AM fungi alone, increased the activity of SOD, CAT and POD. This increase was due to decreased uptake and excess ROS production in leaf tissues, which facilitates the efficiency of these enzymes. *Rhizobium* symbiosis might have activated the activity of antioxidant enzymes due to increase in nutrient availability decrease in Cd content in tissues and secrete phytohormones (Barea et al., 1997; Lenin et al., 2010).

Azcon et al. (2009) observed enhancement of CAT, APX and GR activities in AM fungi inoculated plants, which in turn protected plants from oxidative damage. Toxic metals may also cause oxidative stress and AM fungi buffer the Cd stress through detoxification mechanisms, improve the capability of ROS scavenging and reduce Cd concentration in plants to alleviate Cd stress (Rivera-Becerril et al., 2002; Liu et al., 2011). Arbuscular mycorrhizal fungi are suggested to regulate genes providing protection against ROS (Lanfranco et al., 2005; Saraswat and Rai, 2011).

Although, only a few genes have been identified and characterized, encoding proteins involved putatively in ROS homeostasis in AM fungi. Abad and Khara, (2007) observed greater POD and APX activities in mycorrhizal plants than non-mycorrhizal plants under Cd stress in a wheat plant. Andrade et al., (2008) observed that sunflower (*Helianthus annus* L.) plants associated with *Glomus intraradices* were less sensitive to Cd stress than non-mycorrhizal plants. They further reported that Cd induced POD activity in roots of both mycorrhizal and non-mycorrhizal plants but this increase was much more accentuated in non-mycorrhizal roots. Zhang et al., (2006) observed decreased oxidative stress by intricating antioxidative systems such as POD and non-enzymatic systems in *Vicia faba* grown in soils contaminated with Cd, Pb, Cu and Zn inoculated with *Glomus mosseae*. The correlation study also shows a strong and positive correlation between shoot Cd accumulation with leaf CAT, POX and SOD activity in plants inoculated with *Rhizobium* and/or AM fungi (Figures 5.5, 5.6, 5.10, 5.11, 5.15 and 5.16) grown in soil supplemented with Cd 50 and 100 mg Kg^{-1} soil.

5.2.8 Growth characteristics

The growth parameters decreased with the increasing soil Cd levels in two legumes of lentil and methi (Experiments 2, 3 and 4). The per cent decrease with the age of plants was not as prominent at later growth stages as in early stages, indicates plant acclimatization to Cd stress and probably increased resistance in soil microbial communities such as *Rhizobium* and AM fungi (Paudyal et al., 2007; Wani et al., 2008a). The reduction in growth, biomass and yield with increased levels of Cd has been primarily attributed to decline in photosynthetic pigments and Rubisco activity (Baryla et al., 2001; Bibi and Hussain 2005; Wani et al., 2008) which consequently disturbs the photosynthesis activity (Verma and Dubey, 2002; Wahid et al., 2007). Cadmium is reported to destabilize membrane organization and stability in cells of root which causes water stress due to loss of water and thus increases ABA mediated stomatal closure which causes decrease in transpiration pull. Decreased turgor pressure in meristematic cells negatively regulates the wall extensibility, growth and cell division. Further Cd-induced ABA accumulation suppresses auxin mediated cell growth, which possibly decreased these parameters (Gajewska et al., 2006). These factors, in present experiment, cumulatively led to fresh water loss and decrease in root and shoot length and leaf area of Cd treated legumes. Cadmium treatment also decreased the dry weight accumulation of the tissue of root and shoot. It causes

nutrient deficiency at various levels of absorption from soil and assimilation in plants. Higher accumulation of HMs in soil has dramatic effects on microbial composition of soil and activity their (Khan and Scullion, 2002; Lakzian et al., 2002; Bondarenko et al., 2010). Increasing Cd toxicity results into loss of soil fertility and nutrient levels e.g. Cu, Zn and N level etc. which negatively regulates vital metabolic and physiological functions of plants (Kabata Pendias and Pendias, 2001). Legumes recorded greatest reduction in shoot and root dry weight in HM contaminated sites (Cd, Pb, Zn, Cu) Fatnassi et al., (2014). In legumes, Cd adversely affected the metabolic activities like photosynthesis (Balestrasse et al., 2004; Wani et al., 2006; Noriega et al., 2007), which reduced the dry matter accumulation in roots, shoot and leaf and finally affected the plant biomass production.

Seeds with *Rhizobium* inoculation partially recovered the Cd toxicity in legumes due to metal adsorption and acclimation with age (Experiment 2). Fatnassi et al., (2014) also isolated HM resistant species of *Rhizobium* from the selected legumes grown in contaminated sites. Increased bacterial population provided the legumes adaptive advantage for nitrogenous compounds and substrate for defense proteins through N-fixation.

In the present study, the role of mycorrhiza in the enhancement of plant growth parameters has been reported. These fungi act an efficient sink of excess HMs at the level root and thus reduce their translocation (Garg and Bhandari, 2014). Rabie, (2005) reported a significant stimulatory influence on root and shoot dry weights and inhibitory effects of HM pollutants of both red kidney and wheat plants. Arbuscular mycorrhizal fungi alone improved shoot length and plant dry weight substantially in garden soil as well as waste land soil (Pradhan et al., 2000). Baas Lambers, (1988) found *Glomus fasciculatum* effective in increasing plant growth and shoot-root ratio of *Plantago major*, *Pleiosperma*. A significant increase in fresh weight of *Viburnum dentatum* consequent to inoculation with *Glomus fasciculatum* was found to be more effective than *Glomus macrocarpum* (Verkade and Hamilton, 1987; Verkade, et al., 1988). A significant increase in dry matter yield (Daft and El-Giahmi, 1976; Azcon et al., 2003); phosphate uptake and stimulation of root and shoot growth (Sreeramulu and Bagyaraj, 1999; Pradhan et al., 2000) have also been reported due to mycorrhize. Mycorrhizal symbiosis increases the nutrient acquisition specially provides a large surface area for absorption at low and medium P and N levels (Smith, 2011).

Therefore, improved growth responses seem to be due to AM fungi mediated enriched supply of nutrients and water to the plants favoring the carbon and N metabolism. The importance of mycorrhiza in terms of growth and yield under Cd stress has been reported in *Tagetes erecta* (Ling-Zhi et al., 2011). *Pisum sativum* (Rivera-Becerril et al., 2002), *Thymus ploytrichus* (Wilt field et al., 2004).

From the findings of Experiment 4 it was concluded that dual inoculation was synergistic for plant growth as it decreased Cd accumulation in root and shoot tissues. Increased mineral and further favored the growth morphology of the two legumes. Enhanced root/shoot ratio in AM plants become a barrier in metal transport to shoot (Figure 4.63). Arbuscular mycorrhizal fungi supplies Fe, Cu, Mo and P which is essential for bacterial growth, nodule formation and of root hairs of leguminous plants. Nodules were more in dual inoculated plants (Asimi et al., 1980; Hazarika et al., 2000) as in bean genotypes (Ballesteros-Almanza, 2010) and tree species (Nidhi and Rahangdale, 1999). Franzini et al., (2013) found that interaction of one rhizobial strain R_2 and *Glomus mosseae* had positive interaction in terms of total root dry weight, pod dry weight, total nodule dry weight and nodule numbers under drought stress. Similar effects on plant growth due to combined application of AM fungi and *Rhizobium* was observed under optimal water conditions (Ibijbijen, et al., 1996; Mortimer et al., 2008). Porcel et al., (2006) reported that rhizobial symbiosis affects aquaporin function. Symbiosis of N_2 fixing bacteria and AM fungi enhances plant growth and yield of many legumes (Barea et al., 1992; Azcon and Barea, 2010; Aysan and Demir 2009). A positive and strong correlation between number of nodules per root system vs shoot dry weight was also and nodules per root system vs root dry weight also obtained in plants inoculated with either or both (*Rhizobium* and/or AM fungi) the symbionts (Figures 5.6, 5.11 and 5.16) raised in soil supplied with 50 and 100 mg Cd Kg^{-1} soil.

5.2.9 Yield characteristics

A significant decrease of seed yield of lentil followed by methi plants was recorded when Cd was supplemented to the soil. Heavy metals have been reported to inhibit the photosynthetic efficiency (Verma and Dubey 2002) and hence the concomitant loss of crop yields (Wani et al., 2006; Egharevba and Omoregie, 2010). As discussed above, this reduction is due to the plant genotypic variations to deploy defense mechanism at different levels. Legumes recruit rhizospheric microorganisms to counter exces levels

of soil HMs and to promote their growth responses (Tak et al., 2013) improving the plants nutritional status (Gill and Tuteja, 2011).

The plants inoculated with *Rhizobium* significantly improved the number of pods, number of seeds per pod, weight of 1000 seeds and finally seed yield in both the plants legumes when exposed to increasing soil Cd levels (Experiment 2). The role of *Rhizobium* in phytoremediation, metal detoxification and reclamation of land has also been discussed in literature (Wang et al., 2002; Wei et al., 2010; Hao et al., 2014). Therefore, the tolerance of rhizobia (Maynaud et al., 2013) and increased nutrition (Neumann et al., 1998, Hussain et al., 2012) to plants could potentially facilitate the healthy growth of plants for better yield outputs. Nyemba, (1986) in soybean and Murtaza et al., (2014) in mung bean have shown it to be increased availability of P nutrition and root nodulation.

The effect of application of AM fungi has more encouraging results on the yield of lentil and methi under Cd stress and non-stress conditions, at harvest. Arbuscular mycorrhizal fungi are known, not only to increase plants nutritional status (Hu et al., 2009) but also strengthen the root-*Rhizobium* symbiosis, under the conditions of metal toxicity in soil. Non-mycorrhizal plants have shown to have reduced yield as compared to mycorrhizal plants (Rivera-Becerril et al., 2002, Aloui et al., 2011). The improved yield responses in plants, due to AM fungi association has also been reported by Prayitomo, (2000) and Covacevich et al., (2007).

However, the co-inoculation of two (i.e. *Rhizobium* an AM fungi) had the best result, as compared to single inoculation of either of the two microbes. Abusuwar and Ahmed, (2003) observed the role of mycorrhiza to increase water uptake and P absorption whereas *rhizobial* association work for fixation of atmospheric N and thus both work together to increase food reserves in alfafa crowns and roots to enhance tillering ability of the crop. A strong and direct correlation is obtained between leaf per cent P and N vs seed yield and shoot Cd accumulation vs seed yield in plants inoculated with either or both the symbionts i.e. *Rhizobium* or AM fungi (Figure 5.7, 5.12 and 5.17) raised in soil supplemented with Cd 50 and 100 mg Kg^{-1} soil.

5.2.10 Per cent colonization of AM fungi and toxicity Index

The metal toxicity varies in different plant genotypes growing within a habitat. The genetic variation in plant toxicity index to Cd stress defined by different levels of

tolerance mechanisms deployed at the level of plant to counter the growth repression and support the assimilation of dry weight (Khan et al., 2006; Lux et al., 2011). Cadmium induced differential stress tolerance in five cultivars was also indexed in mustard plants (Gill et al., 2011), in rice, wheat and legumes (Wang et al., 2008b; Wang et al., 2013) and in weeds community (Rather, 2013). Therefore, tolerance index of plants is often calculated as percent decrease of plant dry weight, with increasing toxicity (for instance; here soil mediated Cd toxicity). In the two selected legumes, (Experiment 2) methi recorded the lower toxicity index than lentil when exposed to Cd stress, which could be due to the difference in defense mechanisms employed to counter Cd stress at different levels.

AM fungi inoculation decreased the Cd toxicity and increased tolerance of the plants thus decrease Cd accumulation in plant tissues, change in protein profile (Aloui et al., 2011; Wang et al., 2012) and thus increase the plant biomass production of plants. It has been observed that AM fungi colonized plants showed more tolerance to metals than non-colonized plants (Malekzadeh et al., 2007; Soares and Siqueira, 2008). The fungal inoculation increases retention of metals at the level of root, bind it to the cell wall components (Kapoor and Viraraghavan 1995; Joner et al., 2000) and fungal biomass (Garg and Chandel, 2010) at root level. Binding of HM to chitin in the fungal cell wall reduces its local concentrations in the soil (Sanchez Viveros et al., 2004; Gohre and Paszkowski, 2006). Several organic acids such as citric acid, malic acid and oxalic acid and amino acids are exuded by AM fungi in the rhizosphere which detoxify and resist metal toxicity (Saraswat and Rai, 2011) and enhances root/shoot Cd ratios in AM plants (Gaur and Adholeya, 2004; Soares and Siqueira, 2008; Garg and Chandel, 2010). Other strategies include chelation by compounds such as siderophores and metalothionines (MTs) released by fungi and sequestered by plant-derived phytochelatins (Gaur and Adholeya, 2004).

Inoculation of symbionts induces significant changes in the measured parameters of yield as compared with the uninoculated treatments. Sensitive strains of *Rhizobium* potentially decrease their population, and activity in rhizosphere under Cd stress. Although, proper strains selection of rhizobia could overcome this problem, resulting into slight increase of growth and yield output of legumes. AM fungus, on the other hand, secrete several organic acids, phytochelatins and siderophores to absorb the metal ions and also work as sink for metals. Therefore, AM fungi dilute the

toxic level of HMs in the rhizosphere (Metwally, et al., 2005; Khan et al., 2006). *Rhizobium*-legume symbiosis and availability of nitrogenous compounds support the growth of AM fungi and plant as well. The root response to AM and *Rhizobium* symbioses has been shown to share the signal transduction components (Harrison, 1998; Irfan et al., 2013) therefore, under HM stress dilution of toxicity and availability of nutrients synergistically favor the tripartite association to improve the growth and productivity of legumes. In root-rhizosphere microbial community interact through an array of chemical signaling network (Hassan and Mathesius, 2011). Increased Cd or HM exposure to nodule symbiosis competes with the availability of Mo and Co required for nitrogenase activity induces nodule oxidation and senescence. However, AM fungi work as sinks and protect plant and rhizobia both from excess metal exposure. From the finding of Experiment 4, it was inferred that dual inoculation of legumes with AMF and bacteria resulted in better nutrient acquisition P uptake and remarkable tolerance to HM toxicity and this is in agreements with the findings of Vivas et al., 2003a, b; Muleta, 2010. The elimination of AM fungal populations from soils can have negative repercussions in plant on the establishment of plants and their survival (Ndoye et al., 2015) particularly in legumes growing in toxic areas of HMs (Neumann et al., 1998; Younis, 2007; Wei and Ma, 2010). The rhizobia in roots of legumes facilitate N required for the plant and also AM fungi. Reversibly, AM fungi deliver several inorganic mineral nutrients required by the host plant and rhizobia. Dual inoculation give better AM colonization as compared to application of AM fungi alone in Cd treated legume. It was also showed that the presence of *Rhizobium* in coal wastes and fly ash substrates resulted in significant increase in AM fungal colonization (Wu et al., 2009).

5.3 Conclusions

Conclusively, symbiotic rhizospheric microbes are known to decipher essential role in plant metabolism and augmenting growth and productivity of crops, however a combination of symbionts is required for sustainable agriculture to avoid chemical fertilizers under varied environmental conditions. Legumes display their inherent potential. Methi surpassed other legumes tested and tolerated Cd stress to a significant degree. Lentil was weak in performance and least tolerant to Cd stress among the legumes tested. Therefore, methi exhibited lesser decreases in growth, biochemical and yield characteristics under Cd stress. Correspondingly, methi showed lesser

oxidative stress and increased antioxidant system than lentil to protect photosynthetic machinery and consequent effects on its attributes. *Rhizobium* and AM fungi proved significant potential in the alleviation of Cd stress in both the plants. The decreases in the characteristics observed due to Cd stress were lowered by the application of *Rhizobium* and AM fungi. The co-inoculation of *Rhizobium* and AM fungi not only resulted in restricting the decrease caused by Cd but also nullified the characteristics values over the control. A combined application of *Rhizobium* and AM fungi appears to be most effective in the cultivation of legumes under Cd stress. The effect of this combination was due to the co-inoculation of *Rhizobium* and AM fungi in maintaining plant metabolism and alleviating Cd stress. The combination of plant growth enhancement caused by microbial inoculation in Cd-contaminated soil could be regarded as a promising strategy for remediating metal pollution.

5.4 New reports in the thesis and future prospects

Rhizospheric microbes play a critical role in increasing plants resistance to environmental stress. They not only help in the uptake and assimilation of important plant nutrients in higher plants, and are crucial factors in determining plant growth, vigour and crop yield, but they also alleviate the toxicity and stress caused due to HM. The observations of pot experiments recorded meticulously over three years have no doubt established the alleviation potential of individual as well as combined effects of *Rhizobium* and AM fungi to mitigate the inhibitory effects of Cd on legumes. No effort has been made up till now to study the synergistic and additive impact of these rhizospheric microbes on growth and development of crops under Cd stress. The effect of combined application of *Rhizobium* and AM fungi on oxidative stress and antioxidants under Cd stress has been reported for the first time. The combined application of *Rhizobium* and AM fungi protocol can be recommended to farmers to increase the productivity of legumes under Cd stress.

In addition, an attempt may be made to record the following observations that could not be undertaken due to limited facilities and time.

- To study the mechanism (s) of regulation of symbionts at the molecular level in crop plants under varied environmental conditions.
- To manipulate steps of pathways leading to the production of enzymatic antioxidant (Ascorbate peroxidase, Glutathione reductase and Monodehydro-

ascorbate reductase Ascorbate peroxidase, Glutathione S-transferase), non-enzymatic antioxidant (Ascorbic acid, Glutathione), estimation of H_2O_2 and extent of membrane damage.

- To understand the Cd-induced stress response modulated by *Rhizobium* and AM fungi at molecular and mechanistic levels. This would help to develop an effective strategy to raise transgenic species for stress resistance.
- Same Experiments can be carried out in field conditions.
- Nitrogenase activity for assessing the N_2 fixation ability of legumes can be done.

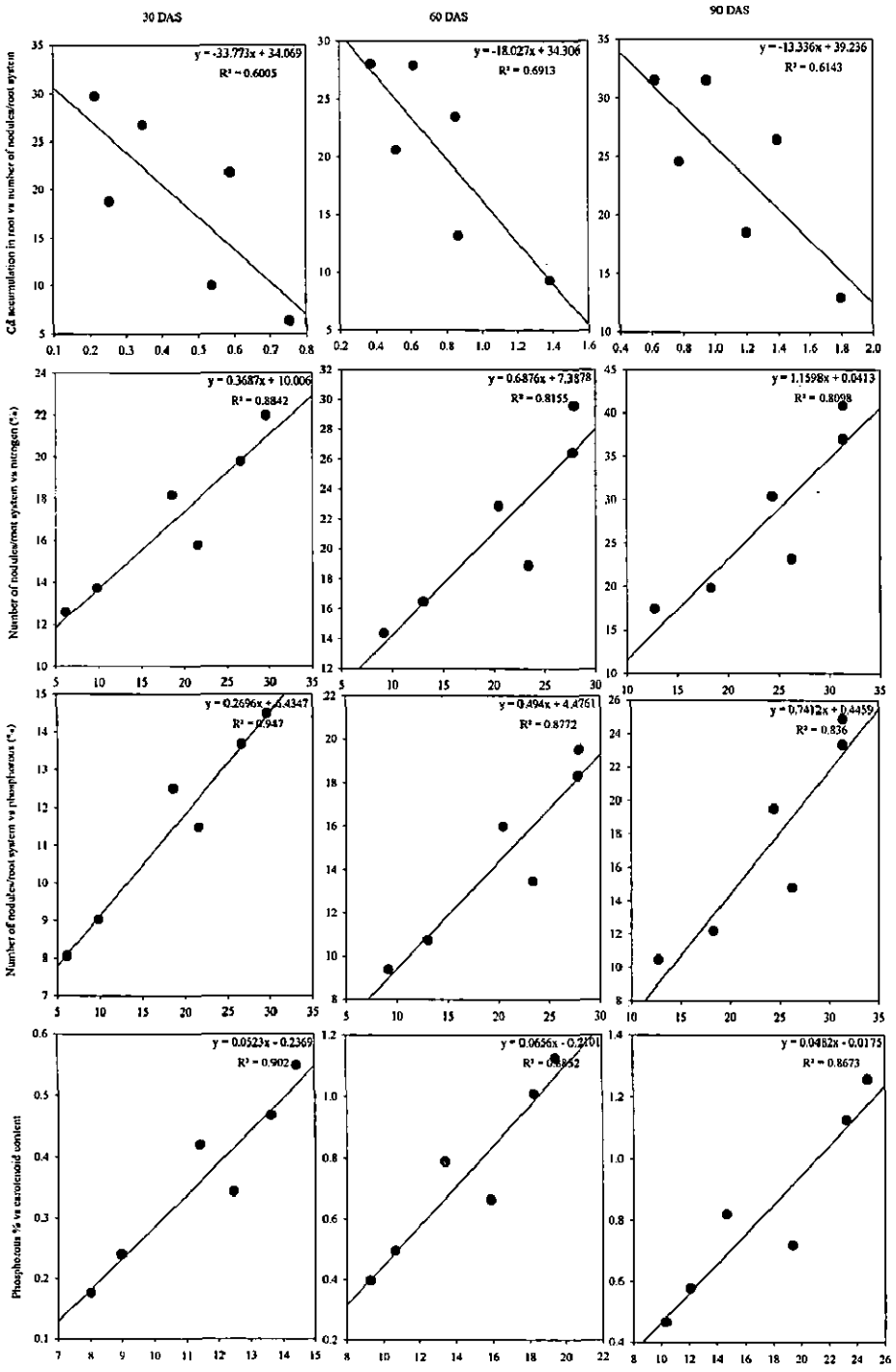


Figure 5.3: Correlation of Cd accumulation in root vs number of nodules per root system, number of nodules per root system vs nitrogen %, number of nodules per root system vs phosphorous % and phosphorous % vs carotenoid content of rhizobial inoculated methi and lentil with 50 and 100 mg Cd Kg^{-1} soil at 30, 60 and 90 days after sowing.

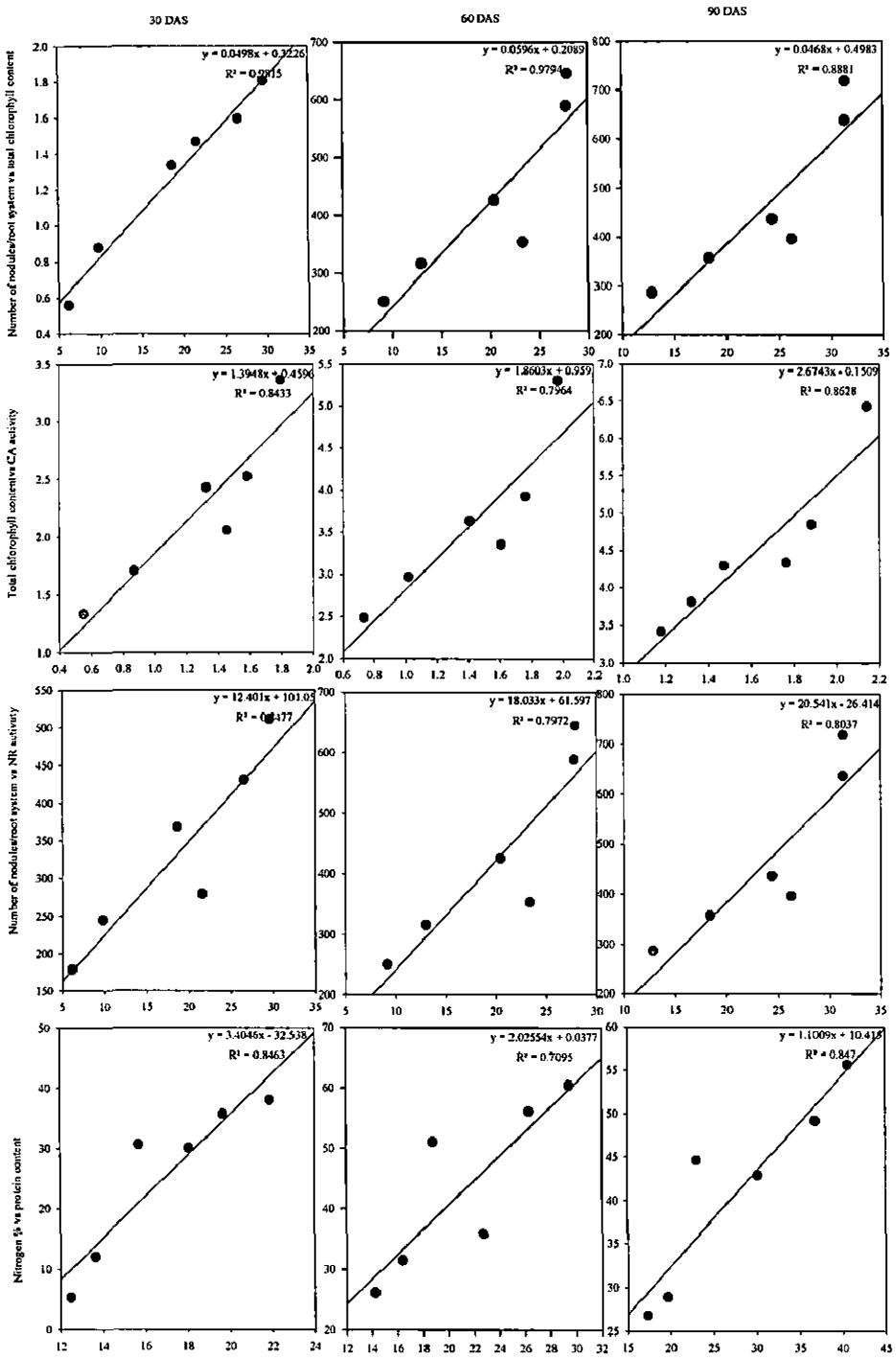


Figure 5.4: Correlation of number of nodules per root system vs total chlorophyll content, total chlorophyll content vs CA activity, number of nodules per root system vs NR activity and nitrogen % vs protein content of rhizobial inoculated methi and lentil with 50 and 100 mg Cd Kg^{-1} soil at 30, 60 and 90 days after sowing.

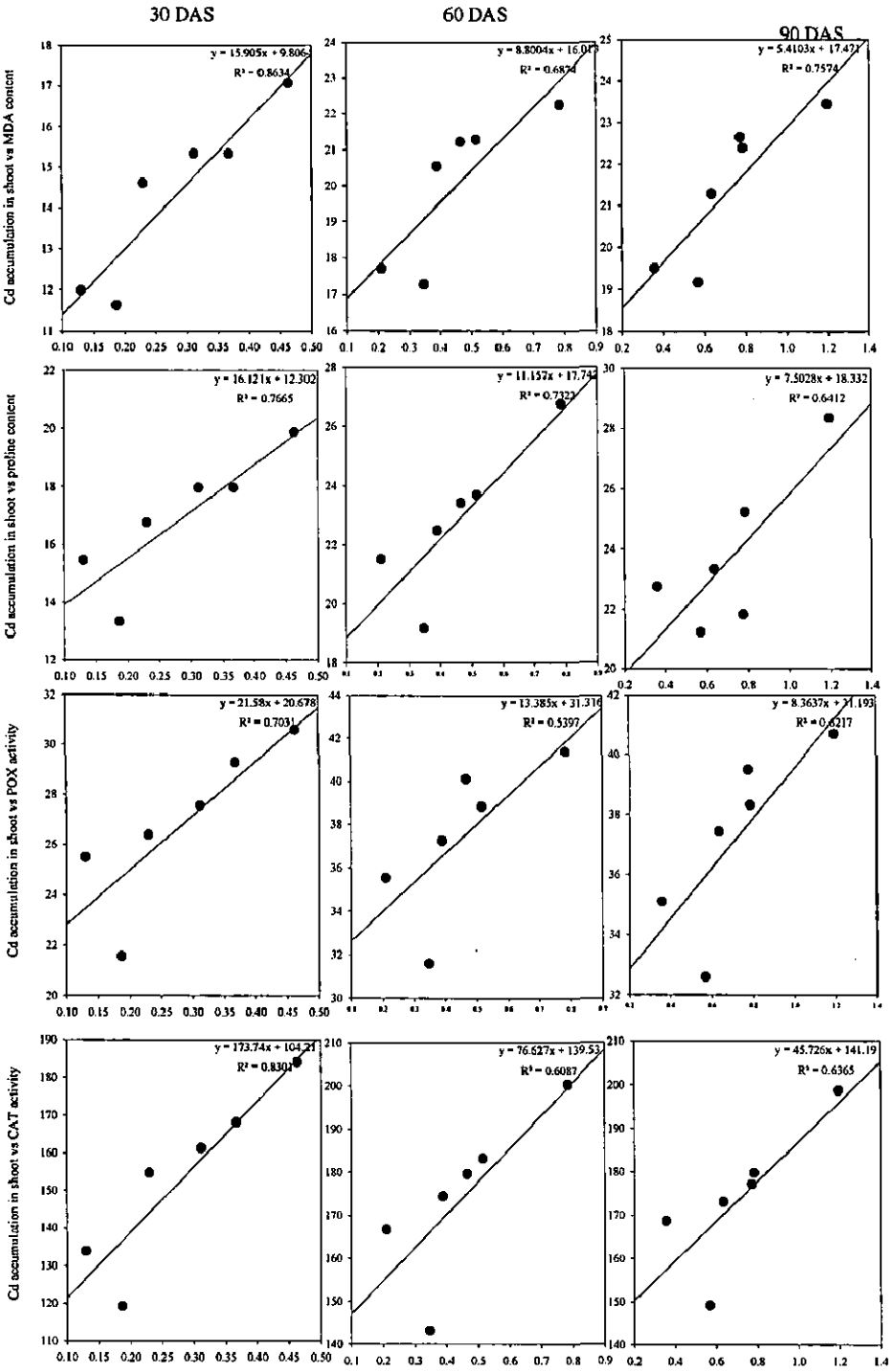


Figure 5.5: Correlation of Cd accumulation in shoot vs MDA content, Cd accumulation in shoot vs proline content, Cd accumulation in shoot vs POX content and Cd accumulation in shoot vs CAT activity of rhizobial inoculated methi and lentil with 50 and 100 mg Cd Kg^{-1} soil at 30, 60 and 90 days after sowing.

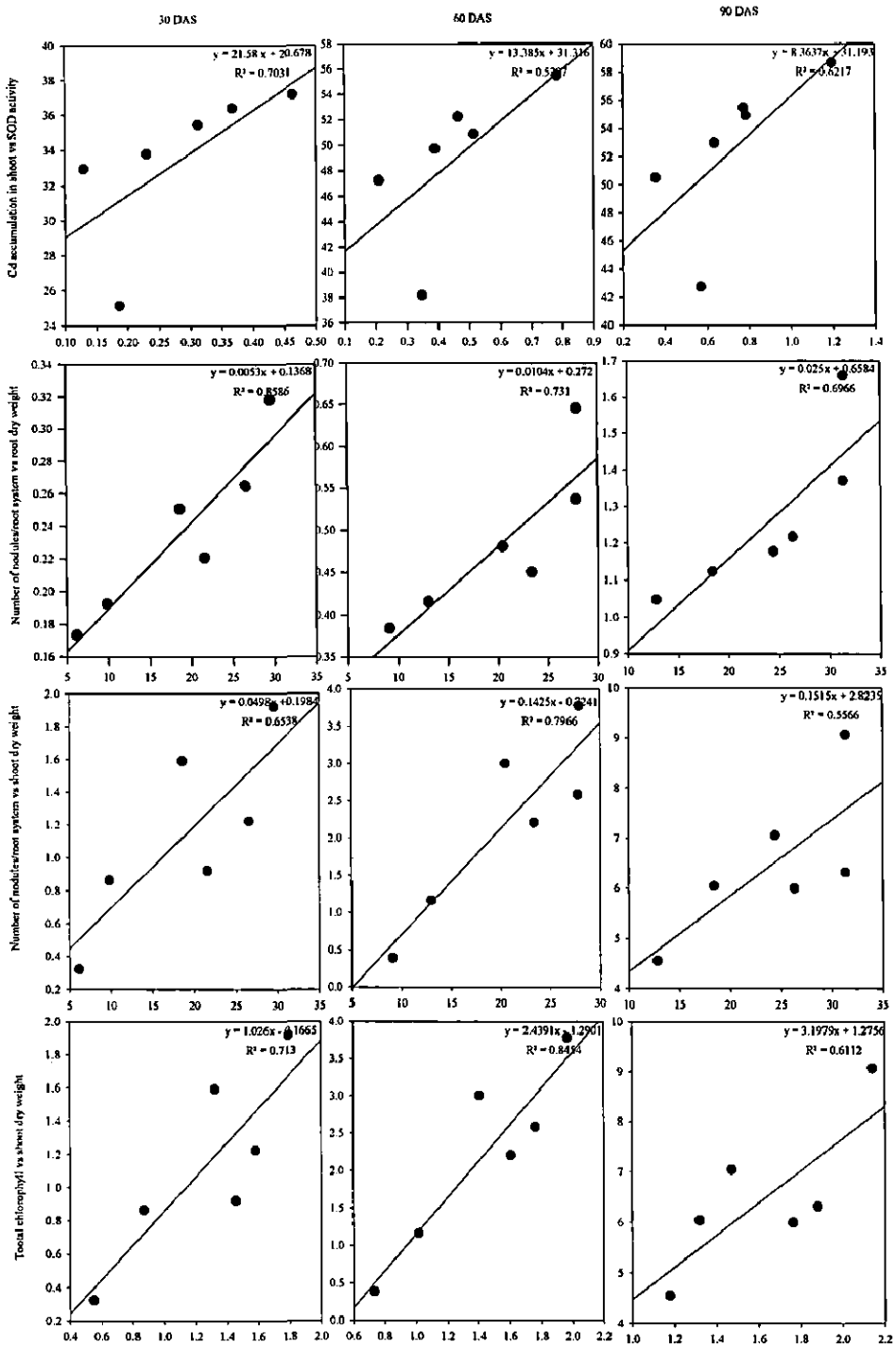


Figure 5.6: Correlation of Cd accumulation in shoot vs SOD content, number of nodules per root system vs root dry weight, number of nodules per root system vs shoot dry weight and total chlorophyll content vs shoot dry weight of rhizobial inoculated methi and lentil with 50 and 100 mg Cd Kg^{-1} soil at 30, 60 and 90 days after sowing.

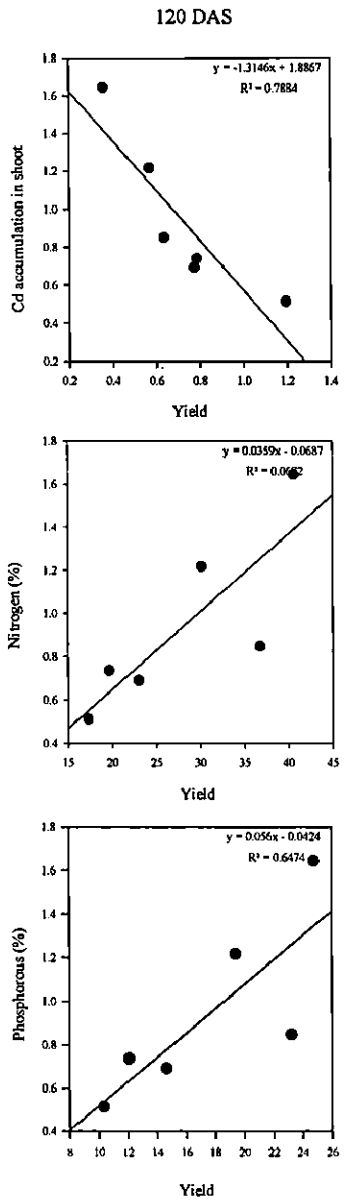


Figure 5.7: Correlation of Cd accumulation in shoot vs yield, nitrogen% vs yield and phosphorous% vs yield of rhizobial inoculated methi and lentil with 50 and 100 mg Cd Kg^{-1} soil at 30, 60 and 90 days after sowing.

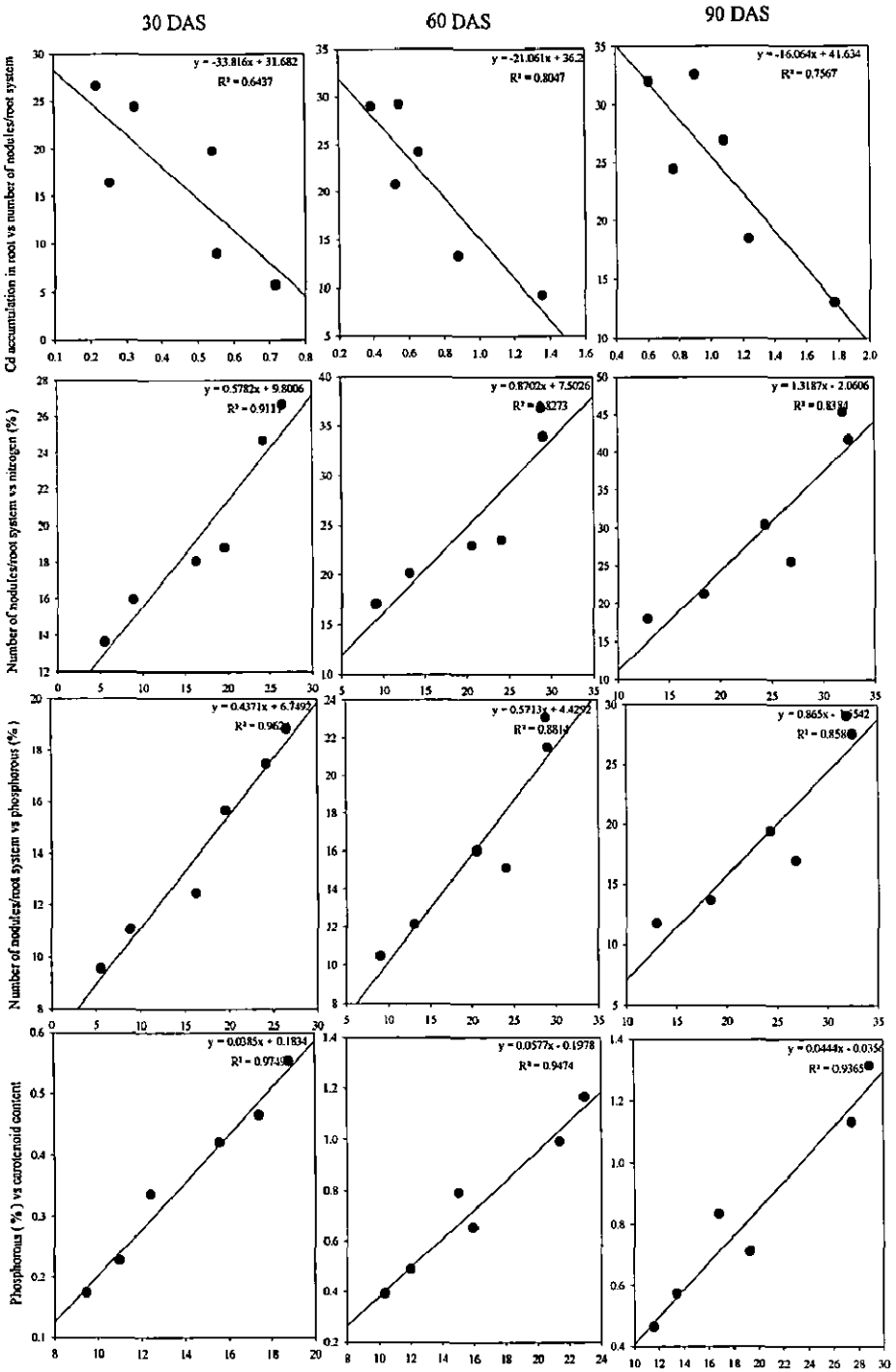


Figure 5.8: Correlation of Cd accumulation in root vs number of nodules per root system, number of nodules per root system vs nitrogen %, number of nodules per root system vs phosphorous % and phosphorous % vs carotenoid content of AM fungi inoculated methi and lentil with 50 and 100 mg Cd Kg^{-1} soil at 30, 60 and 90 days after sowing.

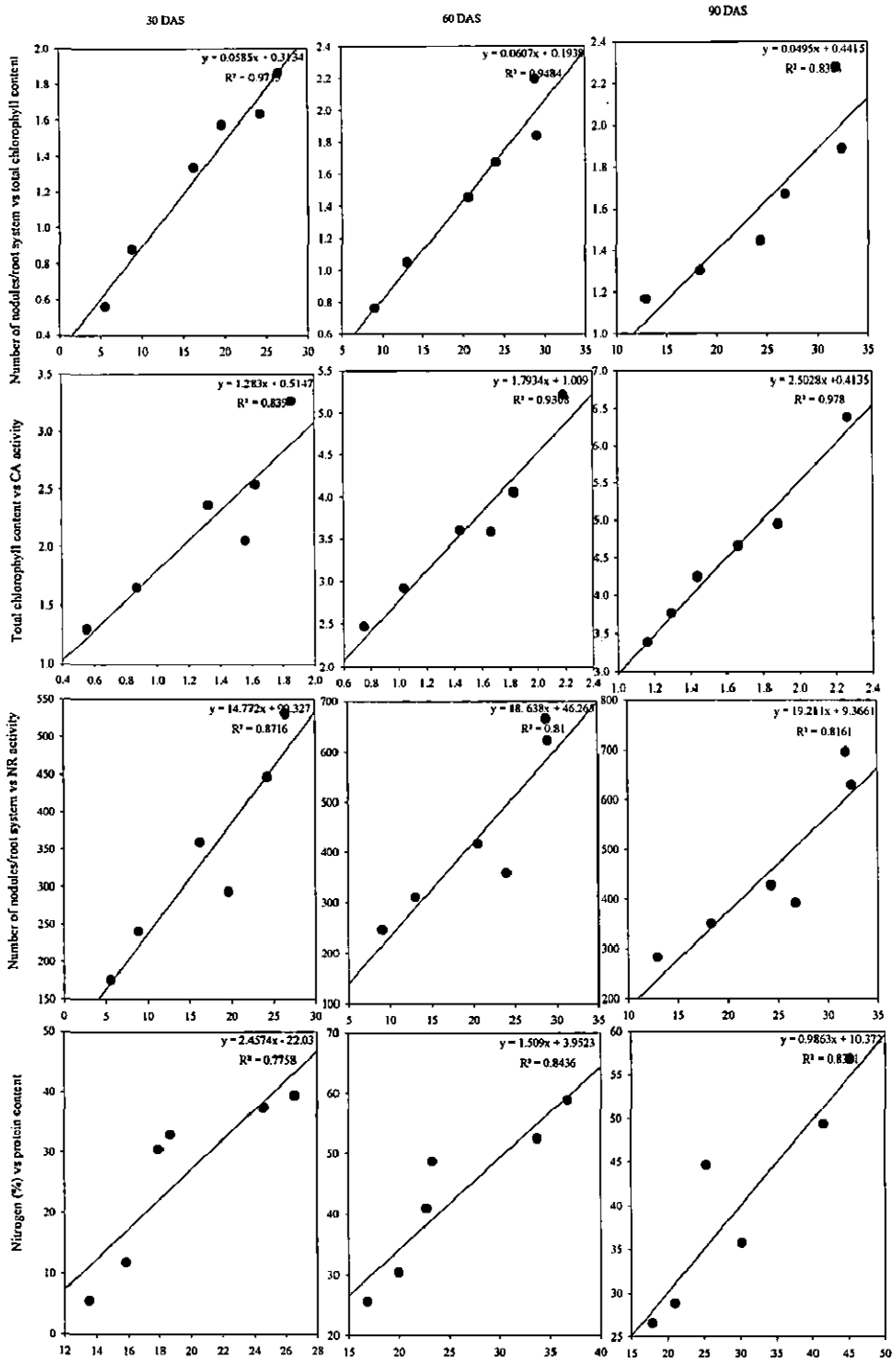


Figure 5.9: Correlation of number of nodules per root system vs total chlorophyll content, total chlorophyll content vs CA activity, number of nodules per root system vs NR activity and nitrogen % vs protein content of AM fungi inoculated methi and lentil with 50 and 100 mg Cd Kg^{-1} soil at 30, 60 and 90 days after sowing.

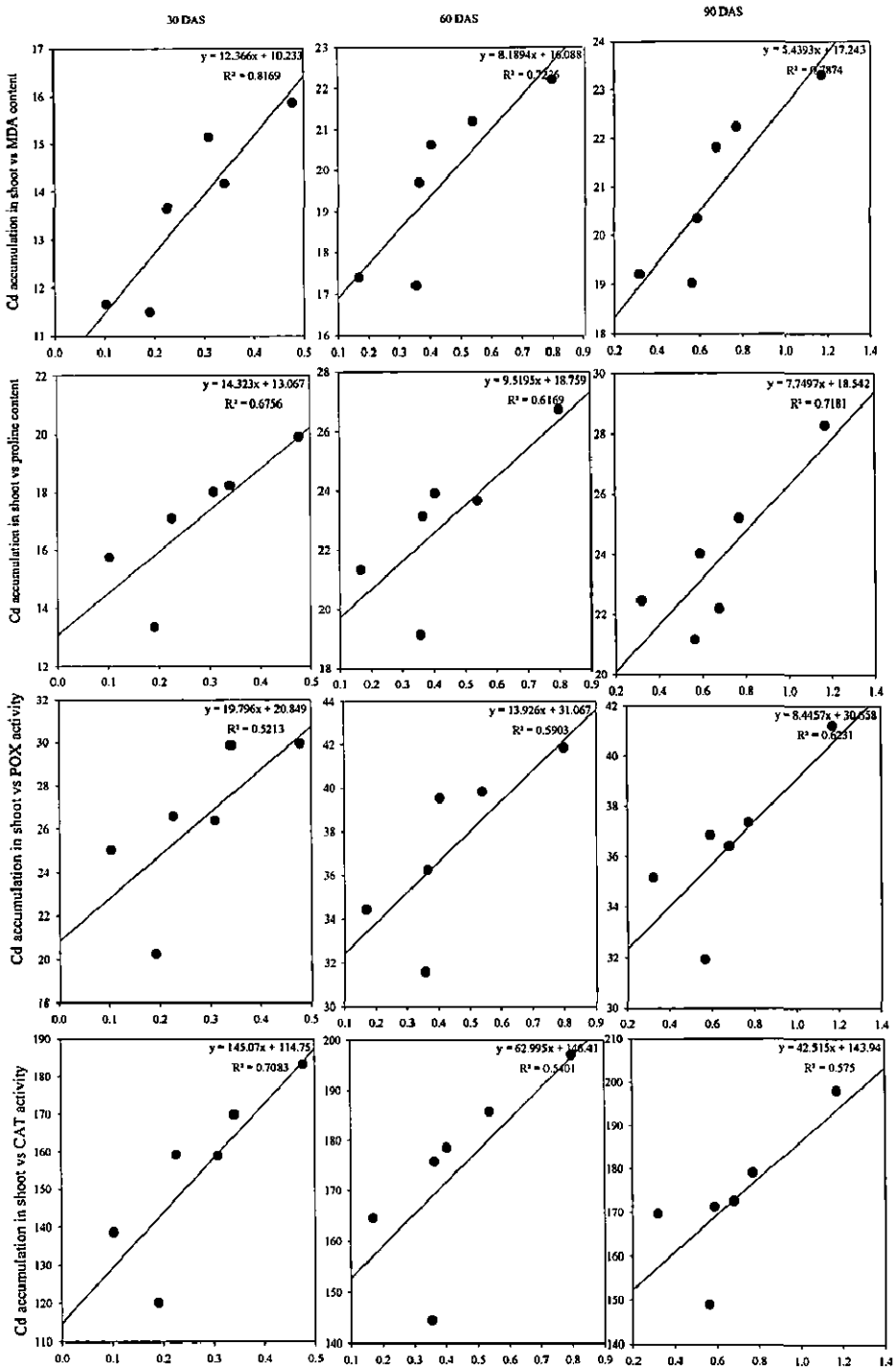


Figure 5.10: Correlation of Cd accumulation in shoot vs MDA content, Cd accumulation in shoot vs proline content, Cd accumulation in shoot vs POX content and Cd accumulation in shoot vs CAT activity of rhizobial inoculated methi and lentil with 50 and 100 mg Cd Kg^{-1} soil at 30, 60 and 90 days after sowing.

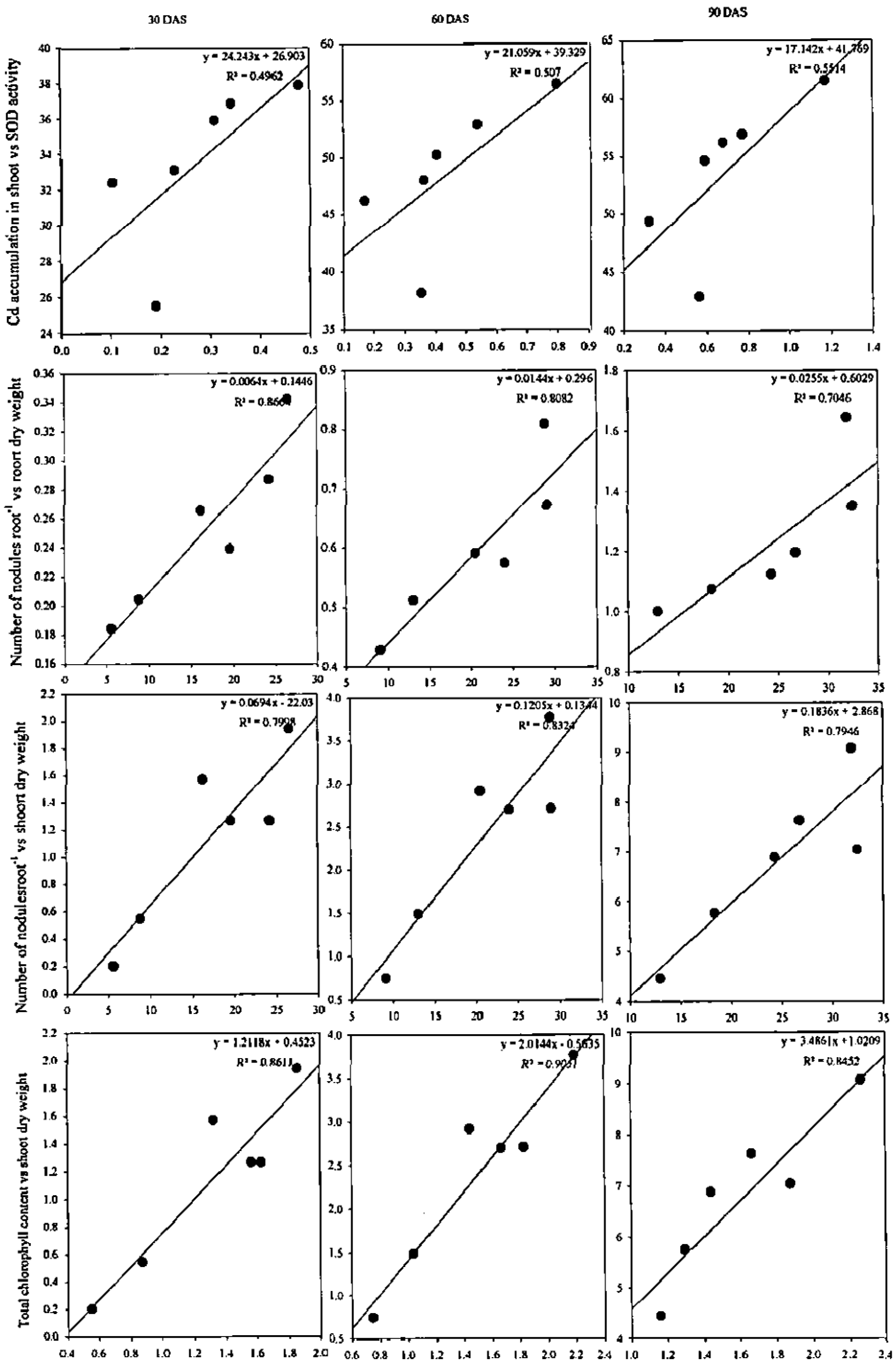


Figure 5.11: Correlation of Cd accumulation in shoot vs SOD content, number of nodules per root system vs root dry weight, number of nodules per root system vs shoot dry weight and total chlorophyll content vs shoot dry weight of AM fungi inoculated methi and lentil with 50 and 100 mg Cd Kg^{-1} soil at 30, 60 and 90 days after sowing.

120 DAS

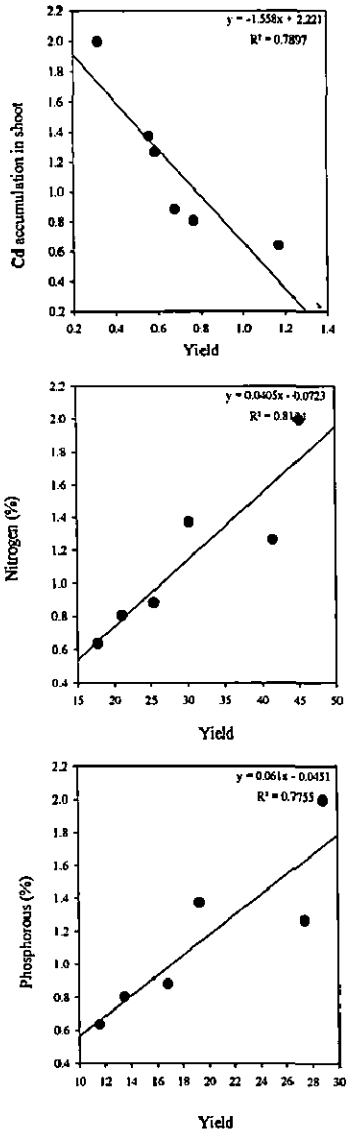


Figure 5.12: Correlation of Cd accumulation in shoot vs yield, nitrogen% vs yield and phosphorous% vs yield of AM fungi inoculated methi and lentil with 50 and 100 mg Cd Kg^{-1} soil at 30, 60 and 90 days after sowing.

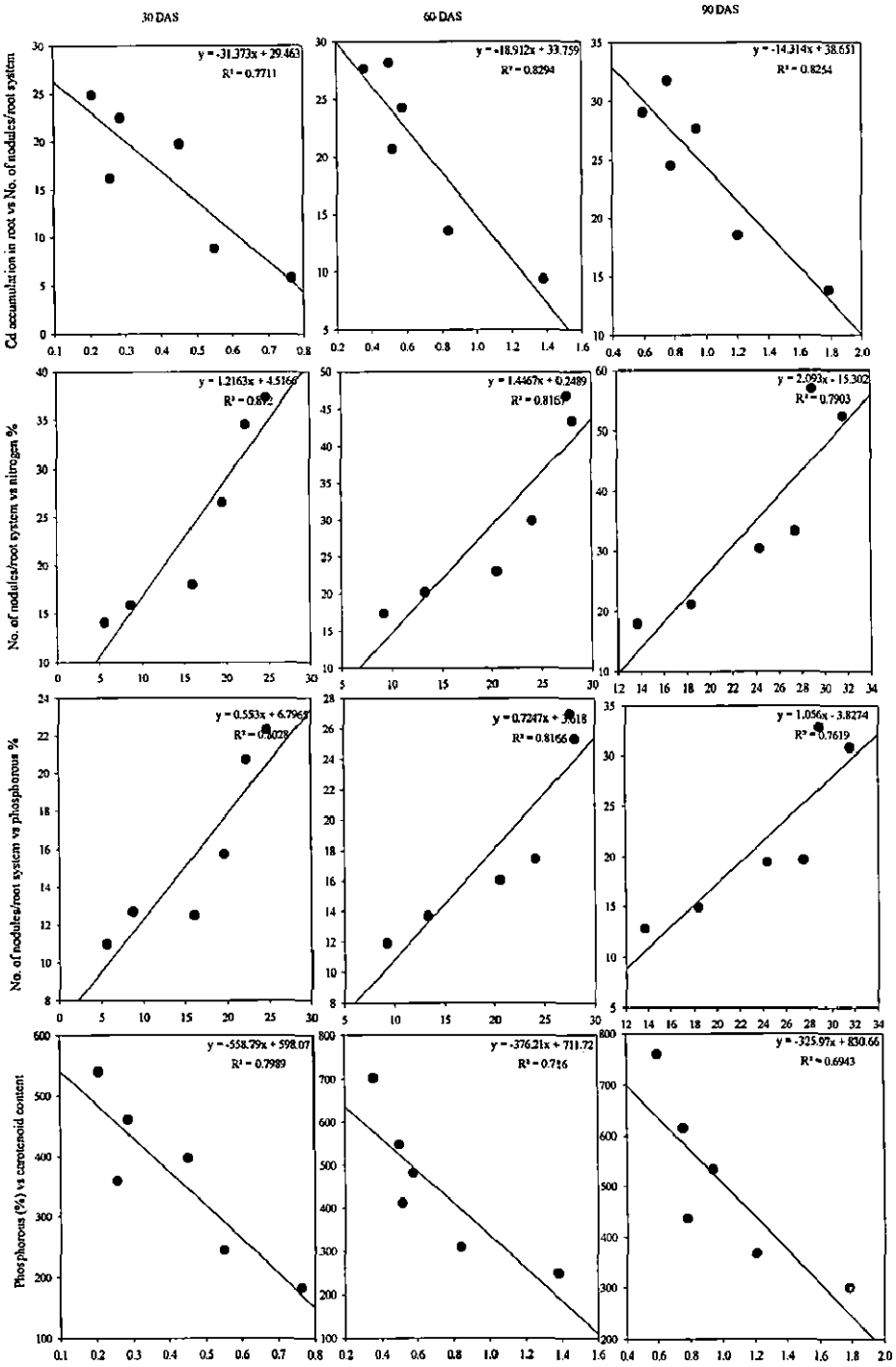


Figure 5.13: Correlation of Cd accumulation in root vs number of nodules per root system, number of nodules per root system vs nitrogen %, number of nodules per root system vs phosphorous % and phosphorous % vs carotenoid content of *Rhizobium* + AM fungi inoculated methi and lentil with 50 and 100 mg Cd Kg^{-1} soil at 30, 60 and 90 days after sowing.

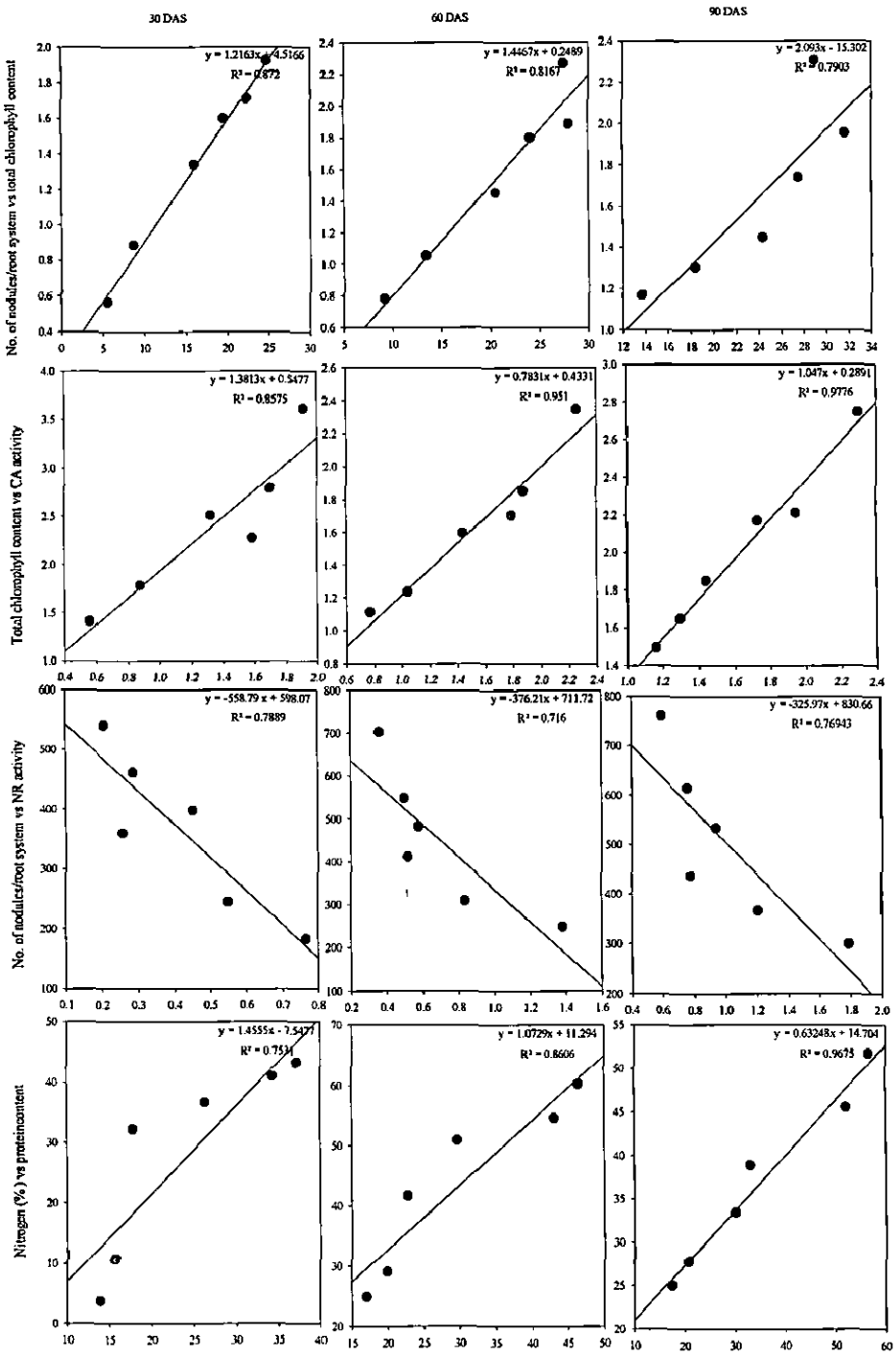


Figure 5.14: Correlation of number of nodules per root system vs total chlorophyll content, total chlorophyll content vs CA activity, number of nodules per root system vs NR activity and nitrogen % vs protein content of *Rhizobium* + AM fungi inoculated methi and lentil with 50 and 100 mg Cd Kg^{-1} soil at 30, 60 and 90 days after sowing.

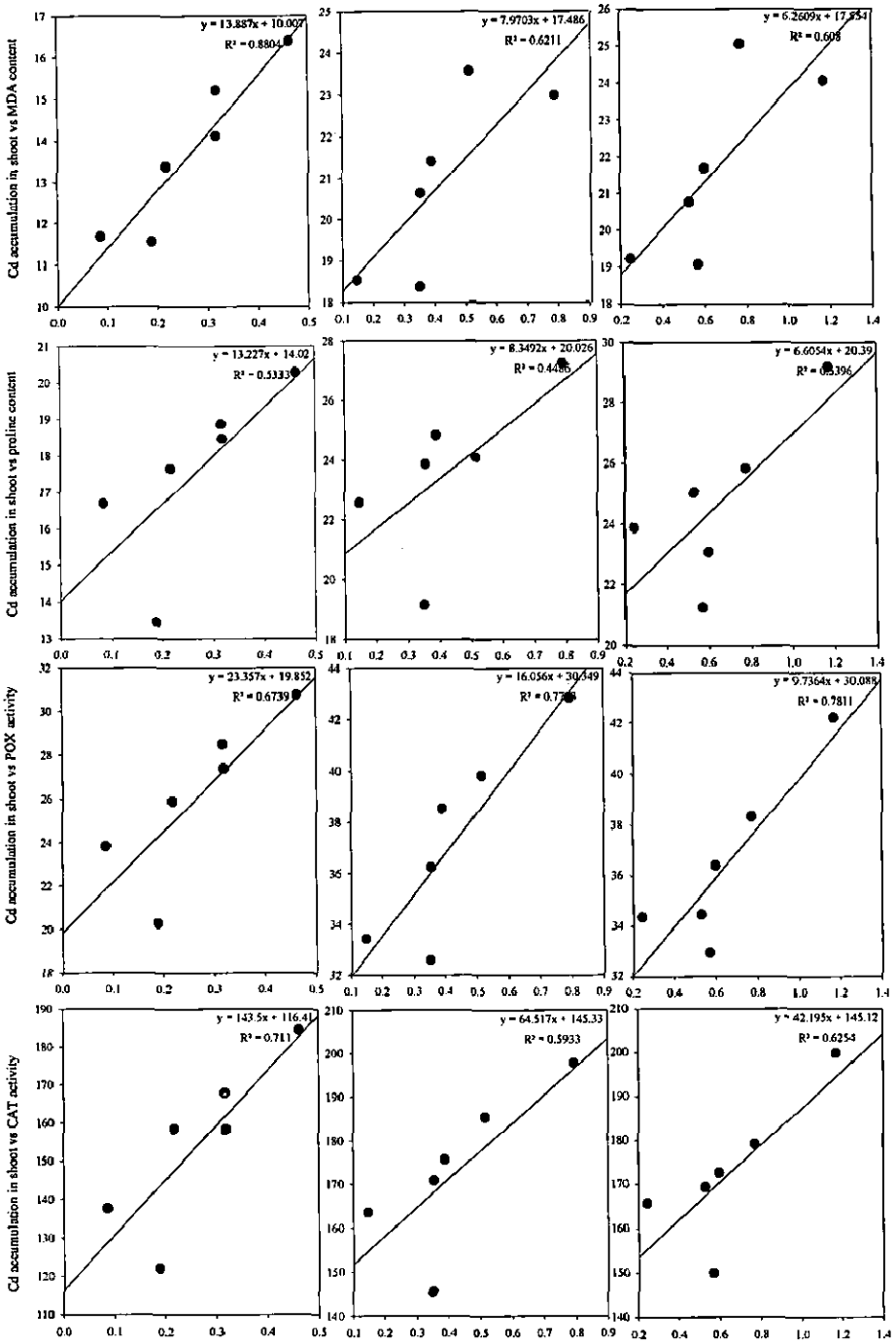


Figure 5.15: Correlation of Cd accumulation in shoot vs MDA content, Cd accumulation in shoot vs proline content, Cd accumulation in shoot vs POX content and Cd accumulation in shoot vs CAT activity of *Rhizobium* + AM fungi inoculated methi and lentil with 50 and 100 mg Cd Kg^{-1} soil at 30, 60 and 90 days after sowing.

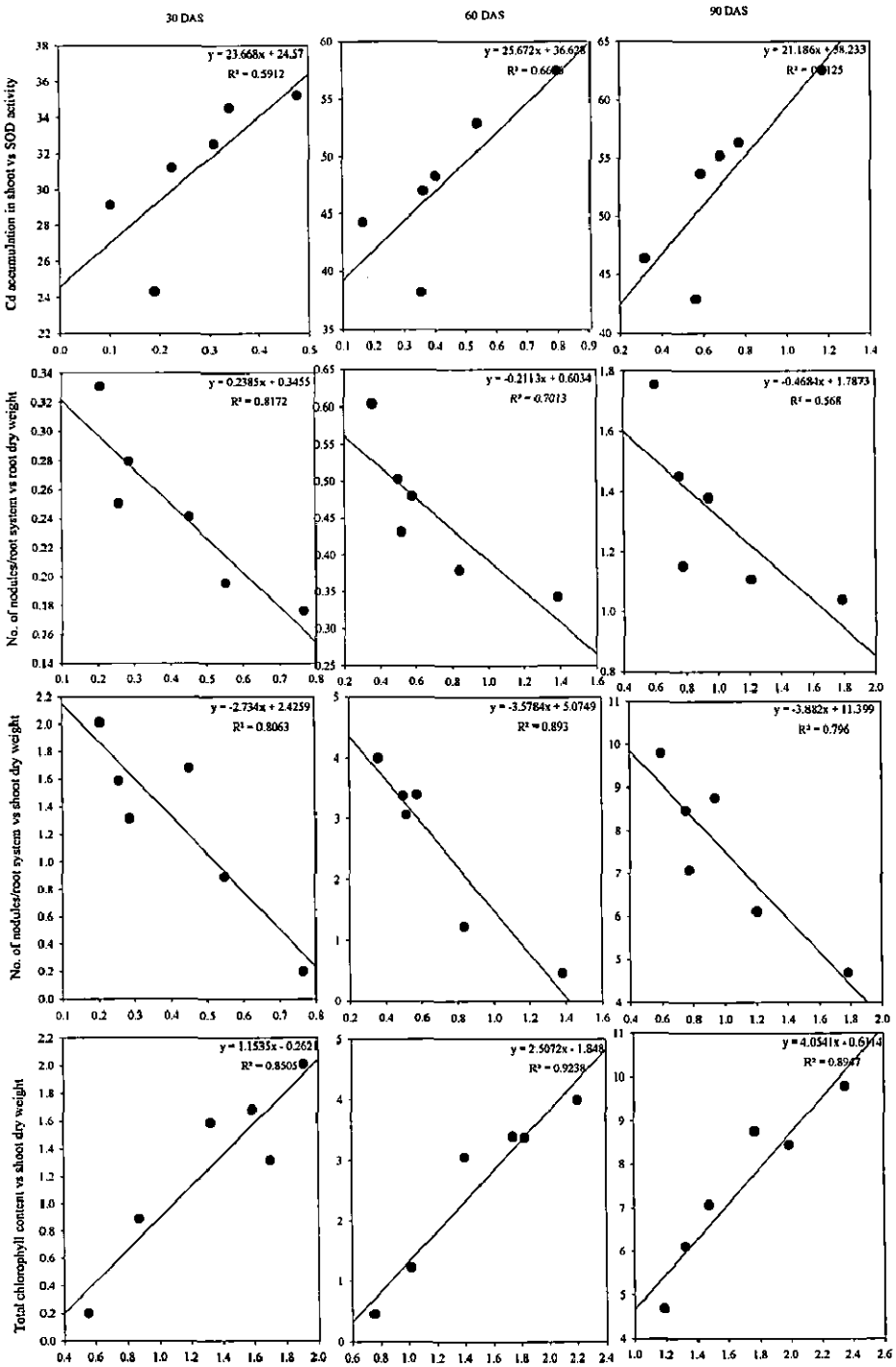


Figure 5.16: Correlation of Cd accumulation in shoot vs SOD content, number of nodules per root system vs root dry weight, number of nodules per root system vs shoot dry weight and total chlorophyll content vs shoot dry weight of *Rhizobium* + AM fungi inoculated methi and lentil with 50 and 100 mg Cd Kg^{-1} soil at 30, 60 and 90 days after sowing.

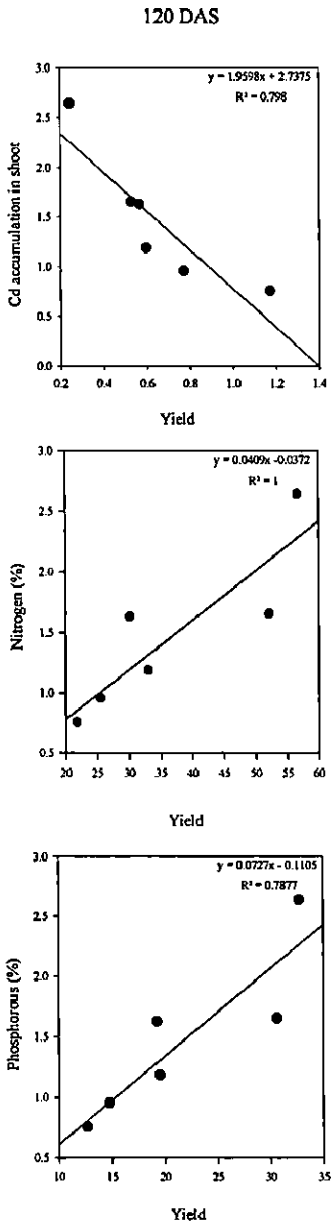
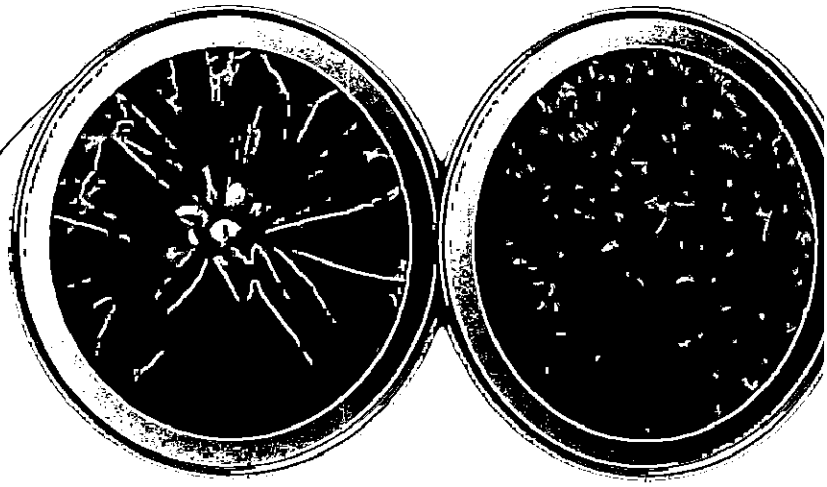


Figure 5.17: Correlation of Cd accumulation in shoot vs yield, nitrogen% vs yield and phosphorous% vs yield of *Rhizobium* + AM fungi inoculated methi and lentil with 50 and 100 mg Cd Kg^{-1} soil at 30, 60 and 90 days after sowing.

Chapter-6

Summary



SUMMARY

The present thesis entitled "Impact of microbial inoculants on cadmium stress in selected leguminous plants." comprises of six chapters.

- **Chapter 1** deals with the importance of the problem and justifications for the present work undertaken were emphasized.
- **Chapter 2** is the review of the literature. It includes the literature available on the aspects of the physiological analysis of various growth, biochemical characteristics, and stress markers, components of antioxidant systems and yield of various crop plants under cadmium (Cd) stress. The importance of inoculation of *Rhizobium* and application of Arbuscular mycorrhizal (AM) fungi in the alleviation and in the regulation of plant growth and development under stress conditions were also reviewed. The chapter has been divided in sections and subsections for the better understanding of the work of other research reports in this field of study. In addition, the critical appraisal of the review of literature has also been included to identify the gap in the field of the study.
- **Chapter 3** includes the details of the material used in the study and the methodology adopted to determine various characteristics recorded in the four experiments have been described in the chapter 3. In addition, also mentions the relevant information on the location of the study and the environmental conditions during the data sampling times.
- **Chapter 4** includes the results of the four experiments. Variation among leguminous crops for sensitivity and non-sensitivity were studied to select Cd-sensitive and non-sensitivite legumes. Details of physiological and biochemical processes occurring in Cd-sensitive and non-sensitive legume and the role of microbes in regulating physiological processes under influence of microbes were studied. The data were statistically analyzed and level of significance was determined at $P<0.05$ using analysis of variance (ANOVA).
- **Chapter 5** discuss the results obtained in the Experiments, in the light of the observations recorded and supported with earlier findings, if available, and also with the help of correlations. This chapter also presents the possible

explanations of the data obtained to reach a conclusion and possible future aspects.

Experiment 1:

This Experiment was performed to study the effect of five concentrations of Cd viz., 0, 25, 50, 75 and 100 mg Kg^{-1} soil on Cd accumulation in plants stress markers, growth, biochemical and yield characteristics of five legume plants viz. methi, broad bean, chick pea, pea and lentil. The treatments in this Experiment were arranged in a factorial randomized block design. Cadmium accumulation in plant, stress markers, growth and biochemical characteristics were studied at pre-flowering (30DAS), flowering (60DAS) and post-flowering (90DAS) stages, while yield characteristics were noted at harvest (120DAS). Tolerance index of five legumes was calculated and the plants were designed as Cd-sensitive and Cd non-sensitive on the basis of their performance under Cd stress. The effects of Cd on growth, biochemical and yield characteristics were found significant at all sampling times. The increase in Cd levels decreases the growth, biochemical and yield characteristics of all the five plants at all sampling stages. The observations showed similar pattern of plants responses to Cd treatments. Maximum reduction in growth, biochemical and yield characteristics was noted with 100 mg Cd Kg^{-1} soil followed by 75, 50 and 25 mg Cd Kg^{-1} soil. Among legumes, methi showed lesser decrease in growth, biochemical and yield characteristics followed by methi, broad bean and chick pea, whereas, pea and lentil exhibited greater reduction in growth characteristics under Cd stress. The tolerance index of cultivars was, methi> broad bean> chick pea> pea> lentil.

Experiment 2:

Experiment 2 was performed on the basis of findings of Experiment 1. As observed in Experiment 1, methi emerged as Cd-least sensitive and lentil as Cd-most sensitive. Among Cd levels, 100 mg Cd Kg^{-1} soil was found to be most toxic and caused maximum reduction in characteristics studied. In the present Experiment, the aim was to study the alleviation potential of *Rhizobium* on the effects of 50 and 100 mg Cd Kg^{-1} soil. The treatments in this Experiment were arranged in a factorial randomized block design. The assessment was done by studying the changes in Cd accumulation in root and shoot, stress markers (MDA and proline content), growth and biochemical characteristics, components of antioxidant defense system and yield characteristics of

non-sensitive (methi) and sensitive (lentil) plants. The sampling stages for Cd accumulation, growth and biochemical characteristics, stress markers, components of antioxidant defense system were determined at pre-flowering (30DAS), flowering (60DAS), and post-flowering (90DAS) stages. The yield characteristics were recorded at the time of harvest (120 DAS). The growth, biochemical characteristics and yield decreased maximally in both the plants treated with 100 mg Cd Kg^{-1} soil. There was significant increase in the Cd accumulation, MDA as well as proline content and components of enzymatic antioxidant system.

Experiment 3:

Experiment 3 was performed on the basis of the findings of Experiment 1. In Experiment 2, it was observed that the application of *Rhizobium* to plants treated with 50 mg Cd Kg^{-1} soil alleviated Cd-induced toxicity in both the plants. Inoculation of *Rhizobium* maximally overcome the toxic effects of 50 mg Cd Kg^{-1} soil in methi (non-sensitive plant) while, the same microbe lowered the Cd-induced toxic effects in lentil to some extent. The present experiment was aimed to study the effect of application of AM fungi for the alleviation of adverse effects of 50 mg Cd Kg^{-1} soil and 100 mg Cd Kg^{-1} soil. The treatments in this Experiment were arranged in a factorial randomized block design. The assessment was done by analyzing the changes in Cd accumulation in root and shoot, growth and biochemical characteristics, stress markers, components of antioxidant defense system were done at pre-flowering (30DAS), flowering (60DAS), and post-flowering (90DAS) stages and yield characteristics was determined at harvest. The alteration in Cd accumulation, growth, biochemical characteristics, stress markers, components of antioxidant defense system and yield characteristics caused by 50 mg Cd Kg^{-1} soil alleviated by AM fungi in methi (non-sensitive plant) and lentil (sensitive plant). Application of AM fungi not only ameliorated the Cd-induced effects but also increased growth, biochemical characteristics, components of antioxidant defense system and thus yield characteristics in methi. In lentil, application of this symbiont only lowered the adverse effects of Cd.

Experiment 4:

Experiment 4 was conducted on the basis of the findings of Experiment 1. In Experiment 2 and 3, it was observed that the application of *Rhizobium* and AM fungi

singly to plants treated with 50 mg Cd Kg^{-1} soil alleviated Cd-induced toxicity in both the plants. Inoculation of both the symbionts maximally overcome the toxic effects of 50 mg Cd Kg^{-1} soil in methi (non-sensitive plant) while, the same microbes lowered the Cd-induced toxic effects in lentil to some extent but it was more than their single inoculation. The present experiment was aimed to study the synergistic effect of dual inoculation of AM fungi and *Rhizobium* for the alleviation of adverse effects of 50 mg Cd Kg^{-1} soil and 100 mg Cd Kg^{-1} soil. The treatments in this Experiment were arranged in a factorial randomized block design. The assessment was done by analyzing the changes in Cd accumulation in root and shoot, growth, biochemical characteristics, stress markers, components of antioxidant defense system and sampling was done at pre-flowering (30DAS), flowering (60DAS), and post-flowering (90DAS) stages and yield characteristics was recorded at harvest (120DAS). The alteration in Cd accumulation, growth, biochemical characteristics, stress markers, components of antioxidant defense system and yield characteristics caused by 50 mg Cd Kg^{-1} soil were alleviated in methi (non-sensitive plant) and lentil (sensitive plant), but the alleviation potential of co-inoculation both the symbionts varied between plants. Combined application of AM fungi and *Rhizobium* not only ameliorated the Cd-induced effects but also increased growth, biochemical characteristics, components of antioxidant defense system and thus yield characteristics in methi. In lentil, application of these symbionts only lowered the adverse effects of Cd. The dual inoculation in all its effects was additive, synergistic and proved superior to their single inoculation.

The present Chapter 6 (Summary) is the resume of the current thesis and followed by the up-to-date references cited in the text. An appendix, containing the methodology employed for the chemical/biochemical analysis, has been described at the end of the thesis.

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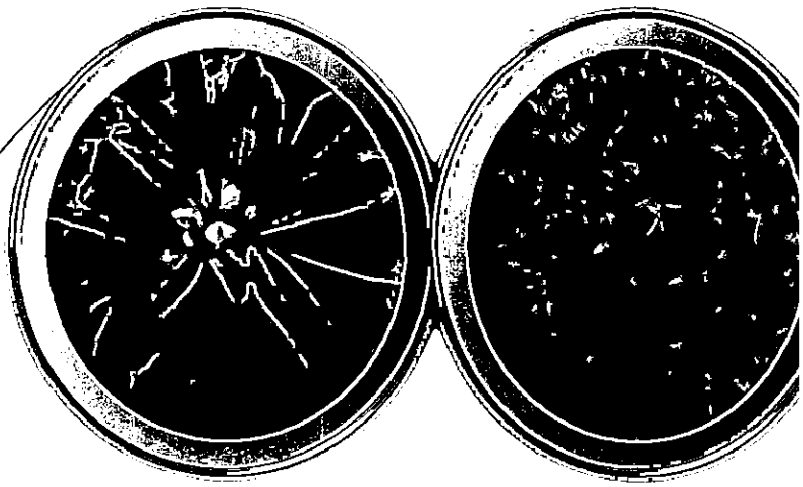

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Appendix


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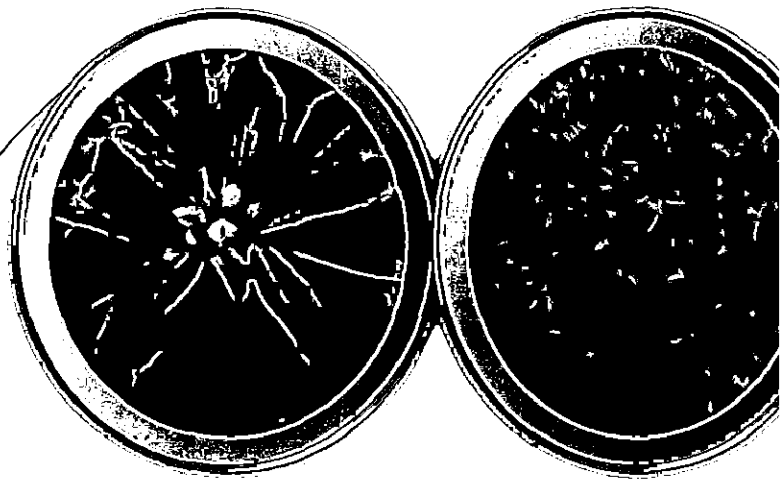

APPENDIX

PREPARATION OF REAGENTS

The reagents for various biochemical determinations were prepared according to the following methods:

1. Preparation of reagents determination of chlorophyll content

1.1 80% acetone

80 ml of acetone was mixed in 20 ml of DDW

2. Preparation of reagents for malondialdehyde level

2.1 Thiobarbituric acid (0.5%)

20% trichloroacetic acid prepared by dissolving 20 ml trichloroacetic acid in 80 ml DDW. Then 0.5 gm 2-Thiobarbituric acid was dissolved in above prepared 20% trichloroacetic acid.

2.2 Trichloroacetic acid (0.1%)

0.1 g trichloroacetic acid dissolved in 100 ml DDW.

2.3 Trichloroacetic Acid (5%)

5 ml of trichloroacetic acid was mixed with 95 ml of DDW.

3. Preparation of reagents for proline estimation

3.1 Sulphosalicylic acid (3%)

3 g of sulphosalicylic acid was dissolved in sufficient DDW and final volume was maintained to100 cm^3, by using DDW.

3.2 Acid ninhydrin solution

1.25 g of ninhydrin was dissolved in a mixture of warm, 30 cm^3 of glacial acetic acid and 6M phosphoric acid (pH 1.0) with agitation till it got dissolved. It was stored at 4°C and used within 24 h.

The 6M phosphoric acid was prepared by mixing 11.8 cm^3 of phosphoric acid with 8.2 cm^3 of DDW.

4. Preparation of reagents for antioxidants

4.1 Potassium Phosphate Buffer (100mM, pH 7.0)

2.72 g of potassium dihydrogen phosphate (KH_2PO_4) and 4.563 g of dipotassium hydrogen phosphate (K_2HPO_4) were dissolved separately in 100 ml DDW. For pH 7.0, mix 39.0 ml of KH_2PO_4 with 61.0 ml of K_2HPO_4.

5. Preparation of reagents for peroxidase estimation

5.1 Pyrogallol phosphate buffer

It was prepared by mixing 25 cm^3 of pyrogallol in 75 cm^3 phosphate buffer (pH 6.0).

6. Preparation of reagents for catalase estimation

6.1 Phosphate buffer (0.1M) for pH 6.8

3.54 g of Na_2HPO_4 was dissolved in 100 cm^3 of DDW and 3.72 g of NaH_2PO_4 was added to 100 cm^3 of DDW. To this 12.3 cm^3 of Na_2HPO_4 was added to 87.7 cm^3 of NaH_2PO_4.

6.2 H_2O_2 (0.1M)

0.34 cm^3 of H_2O_2 was added to 100 cm^3 of distilled water.

6.3 Sulphuric acid (2%)

2 cm^3 of H_2SO_4 was added to 98 cm^3 of DDW.

6.4 0.1N Potassium permanganate

This was made by dissolving 0.162 g of $KMnO_4$ in 500 cm^3 of DDW.

7. Preparation of reagents for superoxide dismutase

7.1 Phosphate buffer (50mM) for pH 7.8

It was prepared by mixing 1.78 g Na_2HPO_4 and 1.56 g of NaH_2PO_4 in 100 cm^3 of DDW separately. 91.5 cm^3 of Na_2HPO_4 with 8.5 cm^3 of NaH_2PO_4 were mixed to get pH 7.8.

7.2 Methionine (13mM)

It was prepared by dissolving 0.193 g of methionine in 100 cm^3 of DDW.

7.3 Nitro blue tetrazolium (NBT) (75µM)

6.13 mg of NBT was dissolved in 100 cm^3 of DDW.

7.4 Riboflavin (2mM)

0.732 mg of riboflavin was dissolved in 100 cm^3 of DDW.

7.5 EDTA (0.1M)

2.92 g EDTA was dissolved in 100 cm^3 of DDW.

8. Preparation of reagents for the estimation of carbonic anhydrase activity

8.1 Cystein hydrochloride solution (0.2M)

48 g cystein hydrochloride was dissolved in sufficient DDW and final volume was made up to 1000 cm^3, by using DDW.

8.2 ***Sodium Phosphate buffer (pH 6.8)***

27.8 g NaH_2PO_4 and 53.65 g Na_2HPO_4 was dissolved separately in sufficient DDW and final volume was made 1000 cm^3. 51 cm^3 of NaH_2PO_4 and 49 cm^3 of Na_2HPO_4 were then mixed to get the required solution.

8.3 ***Alkaline sodium bicarbonate solution***

16.8 g sodium bicarbonate ($NaHCO_3$) was dissolved in aqueous 0.2M NaOH solution [0.8 g NaOH (1000 cm^3)$^{-1}$] and final volume was made up to 1000 cm^3, by using DDW.

8.4 ***0.002% bromothymol blue***

0.002 g of bromothymol blue was dissolved in sufficient DDW and final volume was made up to 1000 cm^3 by using DDW.

8.5 ***0.5N HCl***

4.3 cm^3 of pure HCl was pipetted in sufficient DDW and final volume was made up to 1000 cm^3, by using DDW.

8.6 ***Methyl red indicator***

A pinch of methyl red was dissolved in sufficient ethanol and final volume was made 100 cm^3 using ethanol.

9. Preparation of reagents for nitrate reductase activity

9.1 ***0.1M Phosphate buffer (pH 7.5)***

27.2 g of KH_2PO_4 and 45.63 g of $K_2HPO_4.7H_2O$ were dissolved separately in 1000 cm^3 of DDW. The above solutions of KH_2PO_4 and $K_2HPO_4.7H_2O$ were mixed in the ratio of 16:84.

9.2 ***0.2M KNO_3***

20.2 g of KNO_3 was dissolved in sufficient DDW and final volume was made up to 1000 cm^3, using DDW.

9.3 ***5% Isopropanol***

5 cm^3 of isopropanol was pipetted into sufficient DDW and final volume was made up to 100 cm^3, using DDW.

9.4 ***1% Sulphanilamide***

1 g of sulphanilamide was dissolved in 100 cm^3 of 3N HCl. 3N HCl was prepared by dissolving 25.86 cm^3 of HCl in sufficient DDW and final volume was maintained to 100 cm^3, by using DDW.

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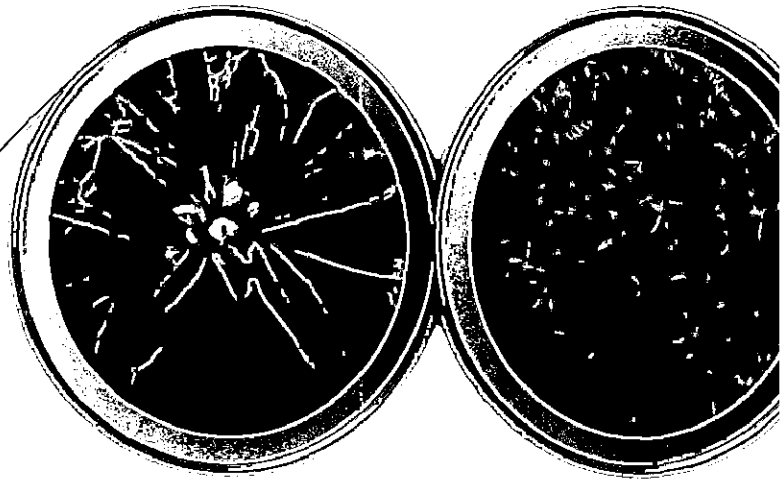

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